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THE PROTEIN C PATHWAY: LINKING INFLAMMATION AND COAGULATION

Desmond Barry White

A thesis submitted for the degree of Doctor in Medicine

University of Dublin

Trinity College

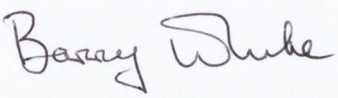
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DEDICATION

This thesis is dedicated to my mother, Eithne White, who has been the major inspiration in my academic career and who has all the attributes of a great scientist.

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ABBREVIATIONS

ATP	Adenosine 5'-triphosphate
AT	Antithrombin
APCD	Acquired protein C deficiency
BSA	Bovine serum albumin
DTT	Dithiothreitol
E coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
FVIIIa	Activted Factor VIII
FVa	Activated Factor V
APC	Activated PC
CVVHDF	Chronic veno-venous haemodiafiltration
cDNA	Complimentary deoxyribonucleic acid
DIC	Disseminated intravascular coagulopathy
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbant assay
FCS	Fetal calf serum
FVIII	Factor VIII
FV	Factor V
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
ICAM 1	Intercellular adhesion molecule 1
IL-1β	Interleukin 1β
IL-2	Interleukin 2
IL-6	Interleukin 6
IL-8	Interleukin 8
Kb	Kilobase
LPS	Lipopolysaccaride
mRNA	messenger RNA
NFκB	Nuclear factor kappa B
PAI-I	Plaminogen activator inhibitor I
PMSF	Phenylmethylsulfonyl Flouride
PBS	Phospate buffered saline
PC	Protein C
PCR	Polymerase chain reaction
Poly(dI-dC)·Poly(dI-dC)	Polynucleotide dI-dC
PS	Protein S
RT	Reverse transcription
RT-PCR	Reverse transcription polymerase chain reaction
RNA	Ribonucleic acid
SD	Standard deviation
SIRS	Systemic inflammatory response syndrome
TNFα	Tumour necrosis factor alpha
TM	Thrombomodulin
TMB	Tetramethylbenzidine

Summary

Activated protein C (APC) is a natural anticoagulant and plays a pivotal role in coagulation homeostasis by inhibiting factors VIIIa and Va. Recent data suggests that APC also negatively regulates pro-inflammatory cytokine production and is protective in animal models of sepsis. The dual functional properties of the protein C (PC) pathway are particularly relevant to severe meningococcaemia which is associated with severe acquired protein C (APCD) deficiency and a high risk of morbidity and mortality. The mortality is primarily caused by cytokine driven multiorgan failure while the major morbidity results from the development of extensive microvascular thromboses (purpura fulminans). Severe APCD is likely to be an integral component in the development of purpura fulminans, however, it may also lead to the loss of an important negative regulator of host inflammatory response and thereby contribute to the mortality associated with cytokine mediated multiorgan failure. The effectiveness of PC replacement therapy in patients with severe APCD, purpura fulminans and multiorgan failure was assessed in an open label study. PC replacement was associated with a reduction in the predicted morbidity and mortality. The decrease in predicted mortality suggests that the cytokine modifying properties of APC may play an important role in the pathophysiology and treatment of multiorgan failure associated with sepsis. The cellular mechanisms responsible for the anti-inflammatory properties of APC were assessed in a THP-1 monocyte cell line. APC resulted in dose dependant inhibition of lipopolysaccharide (LPS) induced TNFα production. This was associated

with a significant reduction in TNFα mRNA. NFκB was then identified as a potential molecular target for APC because it is a critical transcription factor in the generation of TNFα mRNA and a wide variety of pro-inflammatory cytokines, chemokines and adhesion molecules. Pre-treatment with APC resulted in a significant reduction in LPS (100ng/ml) induced activation of NFκB. The ability of APC to inhibit NFκB is likely to be significant given the importance of this transcription factor in the host inflammatory response. However, additional mechanisms must also contribute to the inhibition of TNFα as APC did not inhibit NFκB at higher doses of LPS (10μg/ml) despite significant inhibition of TNFα at both protein and mRNA level.

APC has been shown to be protective in animal models of sepsis and PC replacement therapy in meningococcaemia was associated with a reduction in predicted mortality. This data, together with the ability of APC to inhibit NFκB and TNFα at both protein and mRNA level, is further compelling evidence that the pathways of inflammation and coagulation are intrinsically linked and may have evolved from a common ancestral origin.

Chapter 1

Introduction

Protein C (PC) is a single chain vitamin K dependant protein which is synthesised in the liver and circulates at a plasma concentration of 5 μg/ml. The PC gene is approximately 11 kb long and is located at position q13-q14 on chromosome 2 (Patracchini P *et al*, 1989). PC consists of heavy chain (active site and activation peptide) linked by a disulphide bond to the light chain (γ-carboxylglutamic acid residues and epidermal growth factor regions). Cleavage of the activation peptide by thrombin leads to a conformational change in the protein resulting in the exposure of the active site (Esmon, 1989).

The PC anticoagulant mechanism functions *in vivo* to suppress thrombotic phenomena. This pathway is activated in the microcirculation where activated PC (APC) is generated "on demand" from PC following the binding of thrombin to the endothelial receptor, thrombomodulin (TM) (Esmon, 1989). APC in collaboration with its co-factor, protein S (PS), which circulates free or complexed with the complement regulatory protein C4bBP (60%), inactivates factors Va and VIIIa, which are critical for thrombin generation (Esmon, 1989). A receptor for PC is located on large vessle endothelium and is called the endothelial PC receptor (EPCR) (Fukudome & Esmon, 1994). The EPCR augments PC activation on the endothelium, by bringing it into close proximity to the thrombin:thrombomodulin complex, and is quickly upregulated in response to endotoxin. This response appears to be mediated by thrombin since it is blocked by a specific thrombin inhibitor called hirudin (Esmon *et al,* 1994; Esmon *et al,* 1999). These observations suggest that EPCR plays an important role in the

activation of PC and in the regulation of coagulation homeostasis especially in response to endotoxin. The demonstration that EPCR shares considerable sequence homology with the MHC Class I molecules is further supporting evidence of its role in the inflammatory response (Fukudome & Esmon, 1994).

Activation of PC to APC represents an important host defence mechanism against excessive fibrin formation and when this fails, as is seen in homozygous PC and PS deficiency, neonatal purpura fulminans ensues (Branson *et al,* 1983). This syndrome is characterised by disseminated intravascular coagulopathy (DIC) and necrotic skin lesions and can be prevented by PC replacement therapy (Figure 1.3) (Dreyfus *et al,* 1991). In heterozygous PC deficiency, venous thromboembolic disease in later life is common (Allaart *et al,* 1993). APC may also promote fibrinolysis and has been shown to inhibit plasminogen activator I (PAI-I) *in vitro* (Fourrier *et al*, 1992; Sakata *et al*, 1986). However, this effect could not been reproduced *in vivo* with APC infusions and no abnormalities of fibrinolysis have been detected in patients with inherited PC deficiency (Conard *et al*, 1984).

In addition to its anticoagulant effects, the PC pathway also appears to have potent anti-inflammatory properties. Preloading baboons with APC protects them from lethal infusions of *E. coli* as well as preventing hypercoagulability (Taylor *et al*, 1987). Conversely, the administration of neutralising anti-PC or anti-PS antibodies significantly decreased the dose of *E. Coli* required to attain a lethal effect in this animal model (Esmon *et al*, 1991; Taylor, 1994). More recently, preloading with APC,

in a rabbit model of meningococcal induced septic shock has shown a significant survival advantage in favour of APC therapy (Roback *et al*, 1998). Further evidence for APC anti-inflammatory activity was reported in another *in vivo* model, that of endotoxin-induced pulmonary vascular injury in rats (Murakami *et al*, 1996). Here APC, but not its zymogen PC or active site blocked APC, was able to prevent an increase in vascular permeability and significantly reduced the white cell pulmonary accumulation mediated by endotoxin (Murakami *et al*, 1996). In similar experimental models of sepsis, the administration of different anticoagulants, such as heparin alone or in combination with chloromethyl ketone-inactivated factor Xa, blocked the consumption of fibrinogen but failed to prevent organ dysfunction or improve animal survival, suggesting that the anti-inflammatory properties of APC were independent from the anticoagulant function (Murakami *et al*, 1996; Murakami *et al*, 1997). The dual functional properties of APC are also supported by the fact that concentrations of APC required to exhibit a potential anti-inflammatory effect *in vivo* are insufficient to achieve anticoagulation (Esmon *et al*, 1991)

The protective effects of APC are likely to be mediated, at least in part, by its ability to inhibit lipopolysacharide (LPS) induced TNFα production. *In vivo* studies, using animal models of sepsis, have demonstrated that APC results in a significant reduction in TNFα, and blocking the PC pathway correspondingly increases TNFα production (Esmon *et al*, 1991). The modulation of TNFα production is likely to be significant given the overwhelming experimental evidence to suggest that this cytokine plays a pivotal role in the systemic inflammatory response syndrome (SIRS) (Beutler *et al,*

1985; Waage *et al,* 1987; Waage *et al,* 1991). Since monocytes are the primary site of LPS induced TNFα production, it is likely that the recently described ability of APC to bind to a receptor on monocytes and inhibit LPS induced TNFα and interleukin-1β (IL-1β) production, is a critical component of its anti-inflammatory effect (Grey *et al*, 1994; Hancock *et al*, 1995). To date this receptor has not been characterised. In addition, while the binding of APC to monocytes appears to block the intercellular calcium flux associated with LPS, the cellular mechanisms responsible for the inhibitory effect of APC on TNFα production have not been elucidated (Grey *et al,* 1994; Hancock *et al,* 1995; Murakami *et al,* 1996).

Both the anticoagulant and anti-inflammatory properties of APC are of particular relevance to meningococcal septicaemia where a severe deficiency of PC is associated with the development purpura fulminans and cytokine mediated multiorgan failure (McGehee *et al*, 1967; Heyderman, 1993; Derkx *et al*, 1995). While purpura fulminans induced skin necrosis is the major cause of morbidity, cytokine mediated multiorgan failure is the primary cause of death. The development of severe acquired protein C deficiency (APCD) may not only be responsible for purpura fulminans but may result in the loss of an important negative regulator of the inflammatory response and thereby contribute to cytokine mediated multiorgan failure and death. Case reports have suggested that PC replacement may decrease the morbidity associated with meningococcal induced purpura fulminans, however, this therapeutic option has not been assessed in larger studies (Rintala *et al,* 1998; Rivard *et al,* 1995).

In summary, there is considerable evidence that the PC pathway is an important regulator of the host inflammatory response. The cellular mechanisms responsible for this effect are unknown and the therapeutic potential of PC replacement therapy in meningococcal sepsis has, hitherto, not been fully assessed.



Figure 1.1 Pupura fulminans skin lesion

The characteristic skin lesion associated with purpura fulminans is illustrated in this photograph. The skin rash commences as non-blanching palpable purpuric lesions that subsequently coalesce, darken, blister, and become necrotic. In severe cases gangrene ensues, resulting in a requirement for skin grafting and amputation. Reprinted with permission from Dr. O.P. Smith.

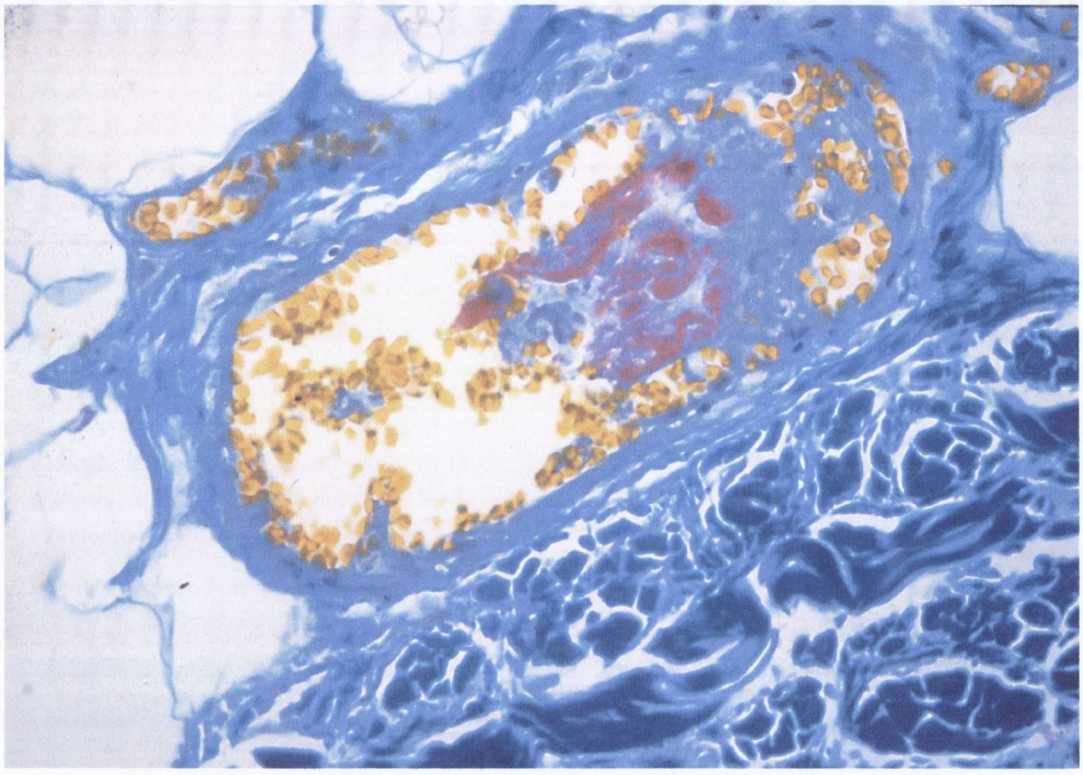


Figure 1.2 Skin biopsy of pupura fulminans

The hallmark histopathogical lesion of purpura fulminans is characterised by dermal vessel thrombosis. Martian Scarlet blue staining illustrates thrombosis in a dermal vessel from a patient with purpura fulminans due to severe meningococcaemia. Fibrin (blue) and platelets (red) are visible within the thrombus. Reprinted with permission from Dr. O.P. Smith.



Figure 1.3 The response to protein C replacement therapy in a patient with pupura fulminans

This picture illustrates the resolution of purpura fulminans due to homozygous protein C deficiency with protein C replacement therapy. Reprinted with permission of Dr. H.P. Scwhartz.

AIMS

1. Clinical assessment of PC replacement therapy in severe meningococcal septicaemia.

2. Determine the effect of APC on LPS induced TNFα production in a THP-1 monocyte cell line.

3. Determine the effect of APC on the LPS induced increase in TNFα mRNA in a THP-1 monocyte cell line.

4. Determine the effect of APC on the LPS induced activation of the critical pro-inflammatory transcription factor, NFκB, in a THP-1 monocyte cell line.

Chapter 2

Introduction

Meningococcal infection is caused by the Gram-negative diplococci, *Neisseria meningitides*. Meningococcal disease can result in a localised infection within the meninges (meningitis) or alternatively may lead to the disseminated form of the illness called meningococcaemia. The majority of patients with meningococcaemia have a benign clinical course, however, a subgroup have a fulminant clinical syndrome which is associated with APCD. Despite recent advances in supportive care this subgroup continue to have a high predicted mortality and morbidity (Havens *et al,* 1989; Giraud *et al,* 1991). The mortality primarily relates to the generation of a wide variety of pro-inflammatory molecules resulting in multiorgan failure (Waage *et al,* 1991; Halstensen *et al,* 1993; Endo *et al,* 1995; Sessler *et al,* 1995; Cowley *et al,* 1994). The major morbidity seen in survivors is caused by the abrupt failure of the PC pathway resulting in purpura fulminans, skin necrosis, skin loss and amputations (Powars *et al,* 1993; Genoff *et al,* 1992). Like other inflammatory response syndromes, meningococcaemia is associated with clinical and laboratory evidence of DIC. However, the reduction in PC activity is far more severe in this particular sepsis syndrome than is seen in other related conditions and is disproportionate to the reductions in PS and AT (Powars *et al*, 1993; Fijnvandraat *et al*, 1995). The precise reasons for the development of severe APCD is not fully understood. It is clear, however, that there is a strong correlation between the severity of APCD, the extent of the thrombotic skin lesions and a negative clinical outcome in meningococcaemia (Powars *et al*, 1993; Fijnvandraat *et al*, 1994).

We hypothesised that early PC replacement therapy in severe meningococcal sepsis would reduce microvascular thrombosis and downregulate the inflammatory response to endotoxin, thereby decreasing both the morbidity and mortality associated with this condition. The effectiveness of PC replacement therapy in severe meningococcal septicaemia associated with APCD and purpura fulminans was assessed in an open label study.

Materials and Methods:

Study subjects

Between January 1996 and June 1999, 36 consecutive patients (17 males; 19 females) with severe meningococcaemia were treated with PC replacement therapy (group I). The mean age +/- sd was 12 +/- 16.4 years (range 3 months to 76 years). Patients were eligible to receive PC replacement therapy if they had a presumptive diagnosis of meningococcaemia with septic shock and purpura fulminans. Septic shock was defined as hypotension (systolic blood pressure < 75 mm Hg for patients <4 years of age and < 80 mm Hg for patients ≥ 4 years of age) which did not correct with fluid resuscitation and required ionotropic support. Purpura fulminans was defined as the presence of extensive purpuric coalescing skin lesions. The Glasgow meningococcal septicaemia prognostic score (GMSPS) (table 2.1 & 2.2) was recorded on each patient at the time of diagnosis and used to predict morbidity and mortality (Sinclair *et al,* 1987; Thomson *et al,* 1991). All patients were treated with conventional antibiotics and fluid resuscitation (40 ml/kg of colloid solution), and required ionotropic support (with adrenaline, noradrenaline, dobutamine or dopamine) and mechanical ventilation. Unfractionated heparin at a dose of 10 - 15 IU/kg per hour was used to maintain the patency of dialysis circuits and to inhibit microvascular thrombus formation. We maintained the platelet count and fibrinogen levels above $50x10^9$/l and 2g/l respectively. A higher than normal threshold was used for fibrinogen concentration because of the use of two anticoagulants, heparin and PC concentrate.

Coagulation parameters were also measured in a control group of 23 patients (12 males; 11 females) who developed meningococcaemia without multi-organ failure or purpura fulminans (group II). The mean age +/- sd was 8 +/- 14 years (range 3 months to 72 years).

Ethical approval was granted by the Local Research and Ethics Committee, and prior consent was obtained from the parents or next of kin.

Protein C concentrate

The PC concentrate used in this study was manufactured by monoclonal antibody purification of viral inactivated prothrombin complex concentrate by Hyland Immuno Baxter (Vienna, Austria). This concentrate undergoes viral inactivation by solvent detergent and vapour heating methods. After reconstitution the concentrate contains 125 IU/ml of PC. One unit is defined as the amount of PC in one millilitre of pooled normal plasma. The concentrate was initially administered intravenously as a test dose of 10 IU/kg over 10 minutes, followed by a loading dose of 100 IU/kg, and a continuous infusion of 10 IU/kg/hr. Thereafter the dose was adjusted on a daily basis with the aim of maintaining a plasma PC level of 80 IU/ml to 120 IU/ml.

Antithrombin (AT) concentrate

Antithrombin concentrate (Atenativ, antithrombin, Kiba Pharmacia) was used in two patients who had plasma AT levels less than 30 IU/ml. The AT concentrate was heat treated and purified by affinity chromatography on heparin sephrous gel.

Laboratory investigations

Venous whole blood was collected into 0.109M sodium citrate (Sarstedt Monovette 9NC/3ml) tubes and the plasma separated by centrifugation at 3800g for 10 minutes. PC and PS were measured by clotting assays (Instrumentation laboratory, Lexicon, MA USA). Antithrombin was measured by chromogenic assay (Instrumentation laboratory, Milan, Italy). The reference ranges in our laboratory for PC, PS and AT were 80-130 IU/ml, 80-140 IU/ml and 70-120 IU/ml respectively. D-dimers were measured by latex agglutination (Fibronsticon, Organon Teknika, Boxtel, NL) and a normal value was defined as <500μg/ml. Fibrinogen was determined by Klauss method (Thromboscreen Pacific Haemostasis) with a reference range 1.5-4g/L. Plasminogen activator inhibitor I (PAI-1) antigen was measured by bio immuno assay (Chromolize ™ PAI-1, Biopool International, Sweden). The reference range for this assay in our laboratory was 4-43 ng/ml.

Results:

A diagnosis of *Neisseria meningitides* was made in all patients, by either PCR on peripheral blood samples or by blood cultures. Protein C and AT were significantly lower in patients who developed multiorgan failure (group I) than in the cohort patients who had meningococcaemia without multiorgan failure or purpura fulminans (group II). The mean +/- sd for PC was 18 +/- 7 versus 41.6 +/- 13.3 IU/ml, $p<0.001$, and AT was 53 +/- 16 versus 81 +/- 20 IU/ml, $p< 0.001$. In addition, there was a statistically significant reduction in plasma PC concentration in comparison to PS and AT within both groups, $p<0.01$ (figure 2.1).

The reduction in plamsa PC levels was inversely proportional to the concentration of D-Dimers, however, plasma PC was still significantly reduced in patients with a normal D-Dimer assay (figure 2.2). The plasma concentrations of AT and PS, at diagnosis and twenty-four hours after admission to hospital, were compared to determine whether the difference in the circulating levels of these natural anticoagulants reflected the shorter half-life of PC. There was only a mild reduction in AT at from 76.9 +/- 24.9 to 67.6 +/- 20.4 IU/ml and PS from 57.3 +/- 17.9 to 52 +/- 15 (figure 2.3). PAI-1 levels were significantly higher in patients with severe meningococcaemia (group I) than those with milder disease (group II), 1222.9 +/- 1319ng/ml versus 185 +/- 296ng/ml, $p = 0.02$ (figure 2.4).

PC concentrate was commenced within 18 hours in 34/36 patients. The mean interval to commencement of PC was 12 hours (range two to 72). Nineteen patients underwent

continuous veno-venous haemodiafiltration (CVVHDF) and two patients underwent peritoneal dialysis. Unfractionated heparin, at therapeutic doses, was administered to 26 of the patients. The reason for not using heparin in the remaining patients was either physician preference or failure to correct the coagulation parameters prior to death. No patient who received unfractionated heparin developed haemorrhagic complications. Antithrombin concentrate was administered in two patients in whom the AT level was less than 30 IU/ml. Both patients made a full and uneventful recovery.

The mean +/- sd GMSPS was 12 +/- 2 which predicted a mortality of 18/36 (50%) (Thomson *et al*, 1991). The actual mortality was 3/36 (8%). One patient died of cerebral oedema secondary to meningoencephalopathy. One patient died within one hour of admission to hospital from refractory hypotension secondary to septic shock. The remaining patient died from intracerebral haemorrhage. The PC and fibrinogen levels were undetectable at diagnosis in this patient and haemorrhage occurred despite replacement therapy with PC concentrate, cryoprecipitate (two units per 10kg body weight of cryopercipitate), fresh frozen plasma 40 (ml/kg), and platelets (post platelet count $90x10^9/l$). The fibrinogen and protein C level were not measured after replacement therapy and before the onset intracerebral haemorrhage. This patient did not receive unfractionated heparin. One patient recovered but suffered irreversible brain damage. This most likely resulted from meningoencephalopthy and/or anoxic injury sustained during a prolonged cardiac arrest, which occurred at time of admission and prior to PC replacement. No haemorrhage was identified on CT scan of the brain.

Four of the 33 patients who survived the acute phase of the illness required amputation (12%). This compares favourably with the predicted risk of amputation of 33% (11/33) (Powars *et al*, 1993; Thomson *et al*, 1991; Kirsch *et al*, 1996; Genoff *et al*, 1992). Two of the patients who required amputation had nonviable limbs prior to the initiation of PC at 48 and 72 hours after admission to hospital. One of these patients also suffered an ischaemic stroke. The amputation rate for patients treated within 24 hours of admission to hospital was 2/31(6.5%). In both cases (patient number 32 and 34), PC was commenced five hours after hospital admission. One of the remaining 28 survivors underwent skin grafting and another patient required chronic haemodialysis. 26/36 (72%) of the patients fully recovered with no complications.

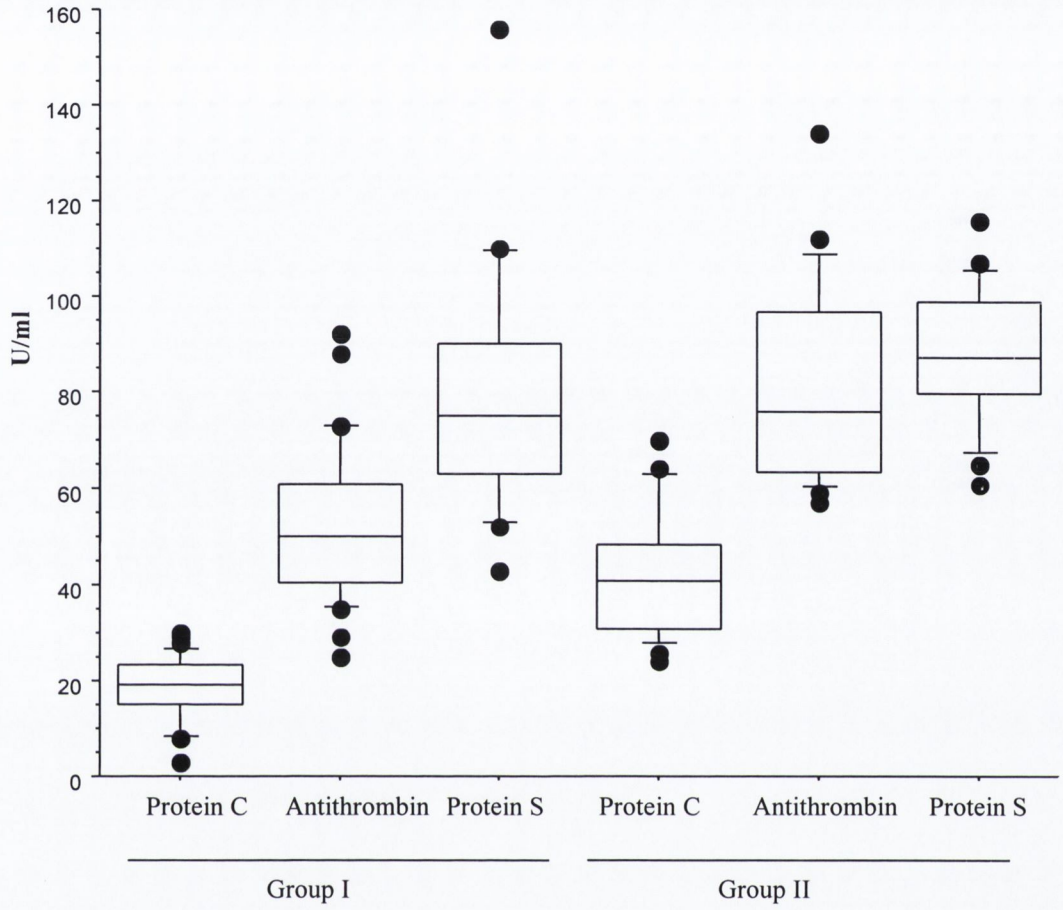


Figure 2.1 Protein C, protein S and antithrombin in meningococcaemia

This diagram represents protein C, antithrombin III and protein S values, obtained at the time of diagnosis, in patients with meningococcaemia who developed multiorgan failure and purpura fulminans (group I) and in patients who did not develop multiorgan failure and purpura fulminans (group II). Protein C was significantly lower than antithrombin III and protein S in group I and group II ($p < 0.001$ and $p < 0.001$ respectively). In addition protein C, and antithrombin levels were significantly lower in group I than group II, $p<0.001$ and $p<0.001$ respectively. Statistical analysis was performed using a Mann Whitney U test.

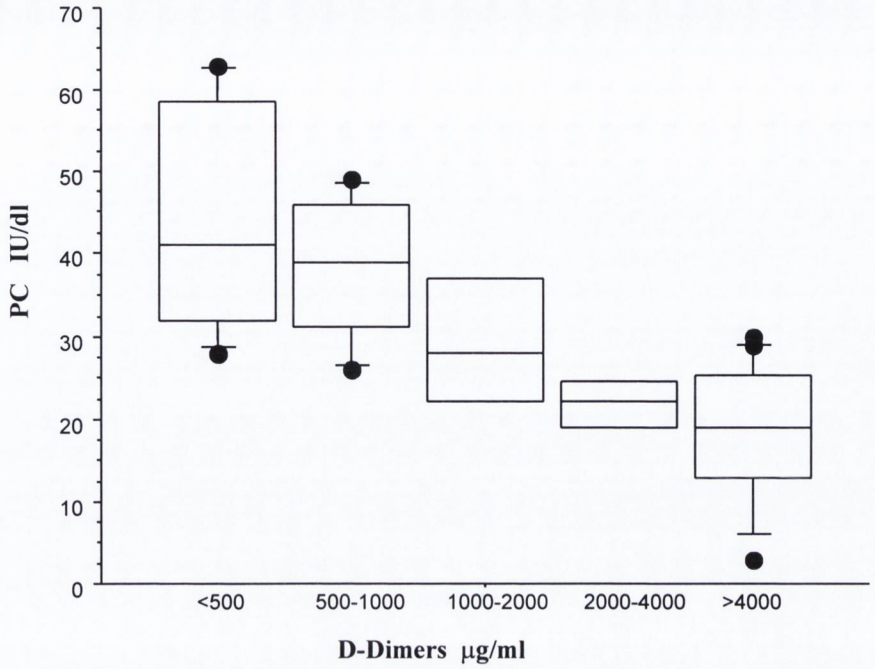


Figure 2.2 The relationship between the concentration of protein C and D-Dimers in meningococcaemia. Plasma protein C and D-Dimers were measured at diagnosis in all patients who developed meningococcaemia with or without multiorgan failure or purpura fulminans (group I and group II). D-Dimers were measured by a semiquantitative latex agglutination assay, and the patients were separated into distinct groups on the basis of D-Dimer values. The mean +/- sd protein C concentrations for patients with D-Dimer values of <500μg/ml, 500-1000μg/ml, 1000-2000μg/ml, 2000-4000μg/ml, >4000μg/ml were 43.8 iu/dl +/-14.35, 38.3 iu/dl +/-9.5, 29.5 iu/dl +/-9.7, 21.7 iu/dl +/-3.3, and 17.76 iu/dl +/-8.5 respectively. While the concentration of PC was inversely proportional to the D-Dimers values, patients with a normal D-Dimer assay (<500μg/ml) still had a significant reduction in protein C. This suggests that consumptive coagulopathy is not solely responsible for acquired protein C deficiency and that other mechanism(s) must also be involved

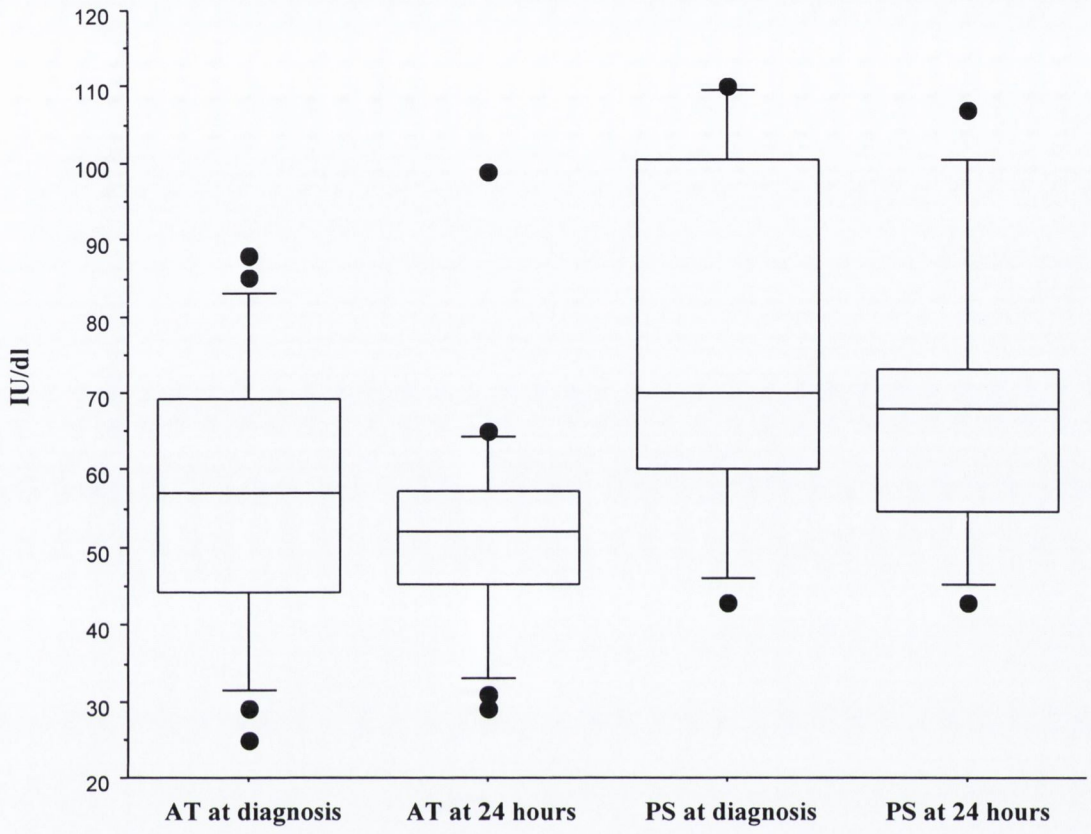


Figure 2.3 Serial changes in protein S and anithrombin in severe meningococcaemia.

This diagram represents the serial changes in antithrombin and protein S values. in patients with severe meningococcaemia who developed multiorgan failure and purpura fulminans and received protein C replacement therapy (Group I). The results at diagnosis were compared to values obtained twenty-four hours after admission to hospital. There was a mild reduction in antithrombin from a mean +/- sd at diagnosis of 57.29 +/-17.9 IU/dl to 52.41 +/- 15 24 IU/dl, however, this failed to achieve statistical significance, p = 0.30. Similarly, there was a mild reduction in protein S from a mean +/- sd at diagnosis of 76.14 +/-24.9 IU/dl to 67+/-20.4 IU/dl, which also failed to achieve statistical significance, p=0.49. This data suggests that the disproportionate reduction in protein C is unlikely to result solely from it's shorter half life. Statistical analysis was performed using a Mann Whitney U test.

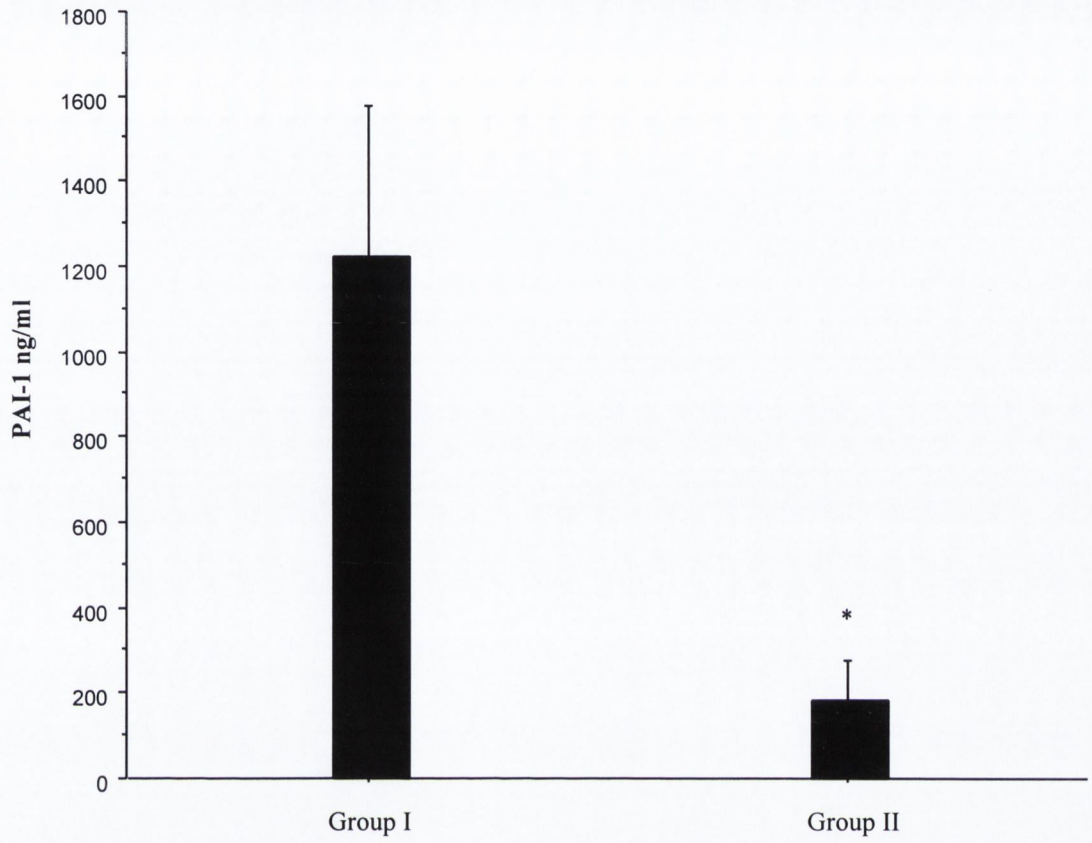


Figure 2.4 The concentration of plasminogen activator inhibitor I (PAI-I) levels in patients with meningococcaemia

PAI-I levels which were measured by immunological assay at the time of diagnosis in patients with meningococcaemia who developed multiorgan failure and purpura fulminans (Group I) and patients who did not develop multiorgan failure and purpura fulminans (group II). PAI-1 was higher in group I (mean +/- sd of 1222.9ng/ml +/-1319) than in group II (mean +/- sd of 185ng/ml +/- 296), *p = 0.02. Statistical analysis was performed using a Mann Whitney U test.

Parameter	Points
Blood pressure <75 mm Hg systolic, age <4 yr Blood pressure <85 mm Hg systolic, age ≥4 yr	3
Skin/rectal temperature difference >3° C	3
Modified coma scale score <8, or deterioration of ≥3 points in 1 h	3
Deterioration in hour before scoring	2
Absence of meningism	2
Extending purpura or widespread ecchymoses	1
Base deficit >8	1
Maximum score	15

Table 2.1. Glasgow meningococcal septicaemia prognostic score (GMSPS)

Parameter	Points
Modified Coma scale	
(i) Eyes open	
spontaneously	4
to speech	3
to pain	2
none	1
(ii) Best verbal response	
orientated	6
words	4
vocal sounds	3
cries	2
none	1
(iii) Best motor response	
obeys commands	6
localizes pain	4
moves to pain	1
none	0

Table 2.2. Glasgow coma score as used in the GMSPS

Patient	AGE (yrs)	Sex	GSMPS	PC	Heparin	Dialysis	Ionotropes	Ventilation	Complications	Outcome
1	18	F	12	20	Y	HF	Y	Y	cerebral edema	dead
2	4	F	9	17	Y	N	Y	Y	nil	alive
3	5	M	11	13	Y	N	Y	Y	nil	alive
4	54	F	15	23	Y	HF	Y	Y	nil	alive
5	11	F	10	25	N	N	Y	Y	nil	alive
6	14	F	12	18	Y	HF	Y	Y	nil	alive
7	2	M	9	9	Y	N	Y	Y	nil	alive
8	15	M	15	0	N	N	Y	Y	Intracerebral bleed	dead
9	8	M	15	3	N	N	Y	Y	Refractory hypotension	dead
10	13	M	11	28	Y	PD	Y	Y	SG	alive
11	18	M	10	19	Y	HF	Y	Y	nil	alive
12	19	M	9	25	Y	HF	Y	Y	nil	alive
13	2	M	14	29	Y	N	Y	Y	nil	alive
14	16	F	15	19	Y	HF	Y	Y	CRF,SG	alive
15	0.3	F	13	21	Y	HF	Y	Y	nil	alive
16	6	M	14	15	Y	HF	Y	Y	nil	alive
17	0.75	M	15	30	Y	HF	Y	Y	nil	alive
18	19	M	9	20	Y	HF	Y	Y	BKA	alive
19	5.3	M	14	18	Y	HF	Y	Y	nil	alive
20	3.3	F	13	15	Y	PD	Y	Y	BKA, ischaemic stroke	alive
21	27	F	11	19	Y	N	Y	Y	nil	alive
22	1	M	8	21	N	N	Y	Y	nil	alive
23	0.66	F	15	19	Y	HF	Y	Y	nil	alive
24	16	F	13	13	Y	HF	Y	Y	nil	alive
25	0.5	M	12	16	N	N	Y	Y	nil	alive
26	0.4	M	12	15	N	N	Y	Y	nil	alive
27	76	F	10	18	Y	HF	Y	Y	nil	alive
28	1.3	M	10	24	N	N	Y	Y	nil	alive
29	5	F	10	20	N	N	Y	Y	nil	alive
30	18	F	12	17	Y	HF	Y	Y	nil	alive
31	6	F	9	29	N	N	Y	Y	nil	alive
32	16	F	12	13	Y	HF	Y	Y	BKA	alive
33	2	F	12	8	Y	HF	Y	Y	Severe brain damage	alive
34	17	M	12	9	Y	HF	Y	Y	BKA	alive
35	17	F	10	26	N	N	Y	Y	nil	alive
36	16	F	12	15	Y	HF	Y	Y	nil	alive

Table 2.3 Clinical details of patients with severe meningococcaemia and purpura fulminans who received PC replacement therapy.
HF = continous veno-venous haemodiafiltration, CRF=chronic renal failure, SG=skin grafting,
BKA =below knee amputation and GMSPS =Glasgow meningococcal septicaemia prognostic score

Parameter	Results
Mean age (range)	12 (3 months - 72 years)
Sex	17 males: 19 females
Mechanical ventilation	36/36
Ionotropic support	36/36
Protein C replacement therapy	36/36
Mean time to PC therapy (range)	12 (2-72 hours)
Antithrombin III replacement therapy	2/36
Unfractionated heparin	26/36
CVVHDF	19/36
Peritoneal dialysis	2/36
Mean GMSPS+/-sd (range)	12+/-2 (8-15)
Predicted mortality	18/36 (50%)
Actual mortality	3/36 (8%)
Predicted amputation rate	10/33 (30%)
Amputation rate	4/33 (12%)
Amputation rate in patients who received PC within 24 hours of admission	2/31 (6.5%)
Skin grafting only	2/33 (6%)
Chronic renal failure requiring dialysis	1/33
Ischaemic stroke	1/33
Severe meningoencephalopathy	1/33
Full recovery with no complications	26/36 (72%)

Table 2.4 Summary of clinical data from patients with severe meningococcaemia and purpura fulminans who were treated with PC concentrate

Discussion

First described by Vieusseaux in 1805, invasive meningococcal disease is a world wide public health problem (Vieussaux, 1805). Localised outbreaks in Ireland, which has one of the highest incidences in Europe, continues to cause serious alarm as a result of the fulminant pattern of disease and higher incidence among children and adolescents. Several scoring systems have been used to predict mortality and morbidity for meningococcaemia. We selected the Glasgow Meningococcal Septicaemia Prognostic Score (GMSPS) (table 2.1 & 2.2) (Sinclair *et al*, 1987). This score is widely used, contains several clinical values plus the base deficit, is quick and easy to perform in most clinical settings and has been retrospectively validated (Thomson *et al*, 1991). In the initial series a score of >8 predicted 100% mortality. More recent reports describe survival at higher scores with a mortality of 30% and 50% for scores of 8 and 12 respectively (Kirsch *et al*, 1996). In addition, a score of 10 or greater is associated with a 30% risk of amputation (Kirsch *et al*, 1996). The mean +/- sd GMSPS in the patients who received PC concentrate was 12 +/- 2, which predicted a mortality of 18/36 (50%). The actual mortality in this cohort was only 3/36 (8%). These patients were treated in eight different hospitals and therefore do not reflect the experience of a single highly resourced centre. Furthermore, the mortality is far lower than previous published data on cohorts of patients which included those with mild, moderate and severe disease (Havens *et al*, 1989; Giraud *et al*, 1991; Fijnvandraat *et al*, 1995; Powars *et al*, 1993; Ramsay *et al*, 1997).

It is unlikely that PC replacement had either a positive or negative impact on the outcome of the patient who died from cerebral oedema secondary to meningoencephalopathy or the one who survived with the severe neurological deficit presumed secondary to meningoencephalopathy and/or anoxic injury. In addition, PC replacement was ineffective in the patient who was premorbid on admission to hospital, which suggests that this therapeutic option may fail to salvage patients who are in the terminal phase of the sepsis syndrome. The only adverse event that may relate to PC replacement therapy was the development of fatal intracerebral haemorrhage. Intracerebral haemorrhage has been previously reported in meningococcal infection (Gironell *et al*, 1995; Seymour & Ferrera, 1998; Huskisson & Hart, 1969; Michieletto & Summonti, 1971). While the mechanism for this complication is unclear, it is likely that the severe deficiency of fibrinogen was an important contributing factor in the patient in this study. The correction of PC to within the normal range would not be expected to increase the risk of bleeding. However, it is possible that PC replacement disturbed a finely balanced equilibrium between severe deficiencies of natural anticoagulants and procoagulants and thereby increased the risk of haemorrhage.

We have demonstrated a lower than expected amputation rate of 4/33 (12%) in patients with severe meningococcaemia and purpura fulminans. This compares favourably with previously published data where 30-50% of survivors with similar disease severity required amputations (Powars *et al*, 1993; Thomson *et al*, 1991; Genoff *et al*, 1992). Furthermore, two of the patients who required amputations already had non-viable limbs at the time of initiation of protein C replacement, 48 and 72 hours after admission

to hospital. The amputation rate for patients treated with PC within 24 hours of admission to hospital of 2/31 (6.5%). These two cases demonstrate that early PC replacement may fail to prevent the morbidity associated with purpura fulminans. It is possible that this failure reflects the biological properties of PC, which primarily prevent clot formation rather than lyse established thrombi. It also possible that the absolute level of PC is not the sole arbitrar in determining either the development of purpura fulminans or response to therapy. This is supported by the absence of purpura fulminans in patients with similar reduction in PC associated with inherited deficiency states (Pabinger *et al*, 1992; Allaart *et al*, 1993). Furthermore, while patients with severe meningococcaemia and purpura fulminans (group I, figure 2.1) have significantly lower protein C levels than those patients with milder disease (group II, figure 2.1), there is considerable overlap between the two groups. Therefore, the rate of reduction in PC or additional defects within the PC anticoagulant pathway may determine the severity of purpura fulminans or the response to PC replacement.

The function of the PC pathway in meningococcaemia may be further compromised by increased C4B binding protein (Fourrier *et al*, 1992) resulting in decreased free protein S, or by a decrease in endogenous activation of PC due to downregulation of endothelial thrombomodulin or an attenuated response in the upregulation of the EPCR to endotoxin (Moore *et al*, 1987; Esmon, 1999; Kurosawa *et al*, 1998). A reduction in free PS has not been reported in severe meningococcal disease and we only detected mild reduction in PS using a clotting based assay which should be sensitive to free PS. In addition, while i*n vitro* studies have demonstrated that endotoxin downregulates

endothelial thrombomodulin, this finding has not been confirmed in animal models of sepsis (Drake *et al*, 1993; Semeraro *et al*, 1993). There is no published data assessing the relationship between rate of EPCR upregulation, in response to endotoxin, and the development of purpura fulminans.

The possibility that patients who develop purpura fulminans harbour an inherited thrombophilic predisposition was recently investigated (Westendorp *et al*, 1997). The prevalence of the genetic risk factors for thrombosis was found to be no higher than expected on the basis of their prevalence in the general population. Other studies have looked at the possible role of the fibrinolytic pathway in meningococcaemia. Plasminogen activator inhibitor 1 (PAI-1) levels appear to correlate with TNFα concentrations and were found to be approximately twice as high in nonsurvivors at a similar TNFα concentration (Kornelisse *et al*, 1996). This is supported by data from the current study in which PAI-1 levels were significantly higher in patients with severe disease in comparison to those with milder disease (figure 2.4). It has yet to be determined whether the elevated PAI-1 levels, seen in patients with severe disease, are a significant contributing factor to adverse outcome. However, if the elevated levels of PAI-1 are associated with inhibition of fibrinolysis then this is likely to be a major disadvantage in the setting of extensive microvascular thrombosis. The clinical importance of PAI-1 is supported by the demonstration that the 4G to 5G polymorphism within the promoter region of PAI-1 gene, which is associated with increased PAI-1 levels, is more prevalent in patients with complicated meningococcal infection (Hermans *et al*, 1999; Westendorp *et al*, 1999). It is likely that the

development of purpura fulminans in meningococcaemia is a consequence of a combination of interactive factors. These may include: activation of the coagulation cascade resulting in increased thrombin generation (Osterud & Flaegstad, 1983); severe APCD; failure of the PC activation mechanisms due to reduction in endothelial thrombomodulin or EPCR; impaired fibrinolysis secondary to increased PAI-1; and poor tissue perfusion due to septic shock which may be exacerbated by protracted use of potent vasoconstrictive agents.

Protein C was reduced to a far greater extent than the other natural anticoagulants, which is consistent with previously published data (figure 2.1) and suggests that this does not solely result from increased consumption. This is supported by the fact that PC was reduced in some patients despite the presence of a normal D-Dimer assay (figure 2.2). It is unlikely that the disproportionate reduction in PC solely reflects it's shorter half life since it has not been described in other sepsis syndromes and a similar reduction in AT or PS was not apparent twenty-four hours after hospital admission (figure 2.3) (McGehee *et al*, 1967; Heyderman, 1993; Fijnvandraat *et al*, 1994). However, the author accepts that the twenty-four levels of AT and PS are likely to be influenced by a variety of confounding variables including ongoing consumption due to DIC and the administration of fresh frozen plasma. Recent data suggests that TNFα inhibits the transcription of PC in liver cells (Yamamoto *et al,*1999) and that PC inhibitors are increased in sepsis (Scully *et al,* 1993). This may explain why PC is reduced to a greater extent than AT or PS and suggests that the high prevalence of APCD in meningococcaemia, in comparison to other infections, may reflect the

magnititude of the inflammatory response. Therefore, the cause of PC deficiency in meningococcaemia is probably multifactorial and likely includes: consumptive coagulopathy; loss from the intravascular space during capillary leak; decreased hepatic synthesis (Yamamoto *et al*, 1999); increased binding to PC inhibitors (Scully MF *et al*, 1993; Marlar *et al*, 1985); and increased ligand binding following upregulation of PC binding sites on mononuclear phagocytes (Hancock *et al*, 1995) and large vessel endothelium (Fukudome & Esmon, 1994; Esmon, 1999).

Unfrationated heparin has traditionally been used in purpura fulminans to prevent the development of microvascular thrombosis and more recently to maintain the patency haemodialysis circuits (Kuppermann *et al*, 1994). In addition to it's anticoagulant properties, heparin has also been shown to downregulate LPS induced TNFα, IL-1β and IL-6 at both mRNA and protein level (Attanasio *et al,* 1998). This anti-inflammatory effect may be responsible, at least in part, for the beneficial effect of heparin therapy in ulcerative colitis (Gaffney *et al*, 1995). It is possible that heparin therapy may improve clinical outcome in severe meningococcal septicaemia by inhibiting thrombus formation and by negatively regulating the host inflammatory response. Despite the widespread use of heparin in purpura fulminans associated with meningococcaemia, this treatment strategy has not been assessed in clinical trials.

There are always hazards in comparing a nonrandomised clinical trial with contemporary and historical controls. Nevertheless, this data strongly suggests that early PC replacement therapy reduces morbidity and mortality in severe meningococcal septicaemia. The improved clinical outcome most likely reflects both the anticoagulant

and anti-inflammatory properties of the PC pathway. Further work is required to define the precise role of PC concentrate in severe meningococcaemia and to identify more effective treatment strategies for patients who fail to respond to replacement therapy.

Chapter 3

Introduction

Few extracellular stimuli provoke systemic responses that are as immediate and profound as those resulting from the endotoxin, also known as LPS, of Gram negative bacteria. Lipopolysaccaride is the prototype activator of cells of the immune and inflammatory systems and may result in the rapid onset of cytokine driven multiorgan failure with high predicted mortality (Taylor *et al,* 1987). While a number of cytokines have been implicated in the pathogenesis of sepsis, several lines of evidence suggest that monocyte derived TNFα is the primary mediator (Beutler *et al,* 1985; Chollet-Martin *et al,* 1992; Waage *et al,* 1987; Tracey *et al,* 1987). Activated PC has been reported to inhibit LPS induced TNFα production in animal models of sepsis, in a THP-1 monocyte cell line and in peripheral blood monocytes (Esmon *et al,* 1991; Grey *et al,* 1994; Hancock *et al,* 1995). We assessed the effect of APC on LPS induced TNFα production in a THP-1 cell line and determined the effects of varying the concentration of fetal calf serum in the culture medium.

Materials and methods

Activated Protein C

Activated PC (lot PCA 162) was provided by Baxter Hyland Immuno, Vienna, Austria. Protein C was isolated from human plasma by immunoaffinity chromatography with anti-PC monoclonal antibody. Activated PC was prepared by activation of immunopurified PC with human thrombin followed by separation by ion exchange chromatography and was certified as sterile and pyrogen free by the manufacturer.

Cell culture

The human monocyte THP-1 cell line (European collection of cell cultures, Salisbury, UK) was cultured at 37°C in 5% CO_2 at a density of 2-9x10^5 per ml. The cell culture medium was RPMI-1640 (Gibco BRL, UK) supplemented with 2mM glutamine (Sigma, UK), 10% fetal calf serum (FCS) (Gibco BRL, UK), penicillin 50units/ml and of streptomycin 50units/ml (Gibco BRL, UK). Cells were counted and >90% viability was confirmed by staining with ethidium bromide acridine orange (Sigma, UK). The cells were then resuspended at a concentration of 2x10^6/ml in culture medium, which contained FCS at varying concentrations (10%, 2%, and 0%). A 2ml cell suspension was transferred to a six well plate and incubated with varying concentrations of APC (final concentrations of 200μg/ml, 100μg/ml and 20μg/ml) ten minutes prior to addition of LPS (*E coli* 0127, Sigma, UK), at a concentration of 100ng/ml or 10μg/ml. The reaction was stopped after four hours by the addition of 2mls of ice cold phosphate buffered saline (PBS) (Gibco BRL, UK). Following centrifugation at 1000g the supernatant was collected and stored at -70°C for subsequent analysis.

Supernatant TNFα was measured using a commercially available enzyme immuno absorbent assay (ELISA) (R&D Systems, UK). 100µl of primary mouse anti-human TNFα antibody at a concentration of 4ng/ml was added to each well of a 96 well microtitre plate (Pharmacia Biotech, UK) for 12 hours. The wells were washed with washing solution (x 3 times) and incubated with 300µl of blocking buffer for 1 hour to occupy non-specific binding sites. The washing solution was prepared from 500µl Tween in one litre of PBS. The blocking buffer was prepared from 40mls of PBS, 2g of sucrose, 0.4g of bovine serum albumin, and 0.02g of sodium azide. The wells were then washed (x 3 times) and 100µl of standards and samples were added and incubated for two hours at room temperature (RT). 100µl of RPMI was added to a control well. The wells were then washed with washing buffer (x 3 times) and 50µl of secondary biotinylated goat anti-human TNFα antibody, at a concentration of 0.2ng/ml, was added and incubated for two hours at RT. Unbound secondary antibody was removed by washing (x 3 times) and 100µl of avidin peroxidase (Sigma, UK), at a concentration of 2ng/µl, was added and incubated for 20 minutes at RT. The wells were washed (x 3 times) and 100µl of Immunopure TMB substrate (Pierce, UK) was added and incubated for 10 minutes at RT. The reaction was stopped with 50µl of 2M H_2SO_4. The presence of TNFα resulted in a colour change from blue to yellow. The absorbency of each well was read at 450nm after a well containing 100µl of Immunopure TMB substrate and 100µl of stop solution was assigned a value of zero. The concentration of the samples was read from a standard curve generated from the absorbance readings of the standards (Table 3.1).

Results

Lipopolysaccharide at a concentration of 10μg/ml was a strong stimulator of TNFα production in THP-1 cells, even in the absence of serum, inducing a 50-60 fold increase in TNFα in comparison to resting cells. The presence of 2% and 10% FCS further enhanced this response with over a 100 fold increase being evident at 10% FCS. Lipopolysaccharide at 100ng/ml also strongly stimulated TNFα production resulting in a 50 fold increase in the presence of 10% FCS (figure 3.1). Activated PC resulted in significant inhibition of TNFα production. The most marked inhibition was seen at a concentration of APC of 200μg/ml, which blocked the effect of 100ng/ml LPS by 75.3+/-2.9%. The inhibitory effects of APC were less marked at the higher concentration of LPS (10μg/ml) and in the presence of FCS, however, APC 200μg/ml resulted in at least a 50% reduction in TNFα under all conditions tested (figure 3.1). There was no reduction in TNFα activity when standards, diluted in cell culture medium, were incubated with APC or PC at 200μg/ml for four hours.

Dilution		Final concentrations
Tube 1	1000µl of TNFα at a concentration 2000pg/ml of	2000pg/ml
Tube 2	500µl from tube 1 + 500µl RPMI	1000pg/ml
Tube 3	500µl from tube 2 + 500µl RPMI	500pg/ml
Tube 4	500µl from tube 3 + 500µl RPMI	250pg/ml
Tube 5	500µl from tube 4 + 500µl RPMI	125pg/ml
Tube 6	500µl from tube 5 + 500µl RPMI	62.5pg/ml
Tube 7	500µl from tube 6 + 500µl RPMI	31.25pg/ml
Tube 8	500µl of RPMI	0 pg/ml

Table 3.1. Preparation of TNFα standards

TNFα standard provided by the manufacturer was reconstituted to 6µg/ml with standard diluent buffer and further diluted in phosphate buffered saline (PBS) to give a final concentration of 2000pg/ml. TNFα standards were prepared by serial dilutions with RPMI.

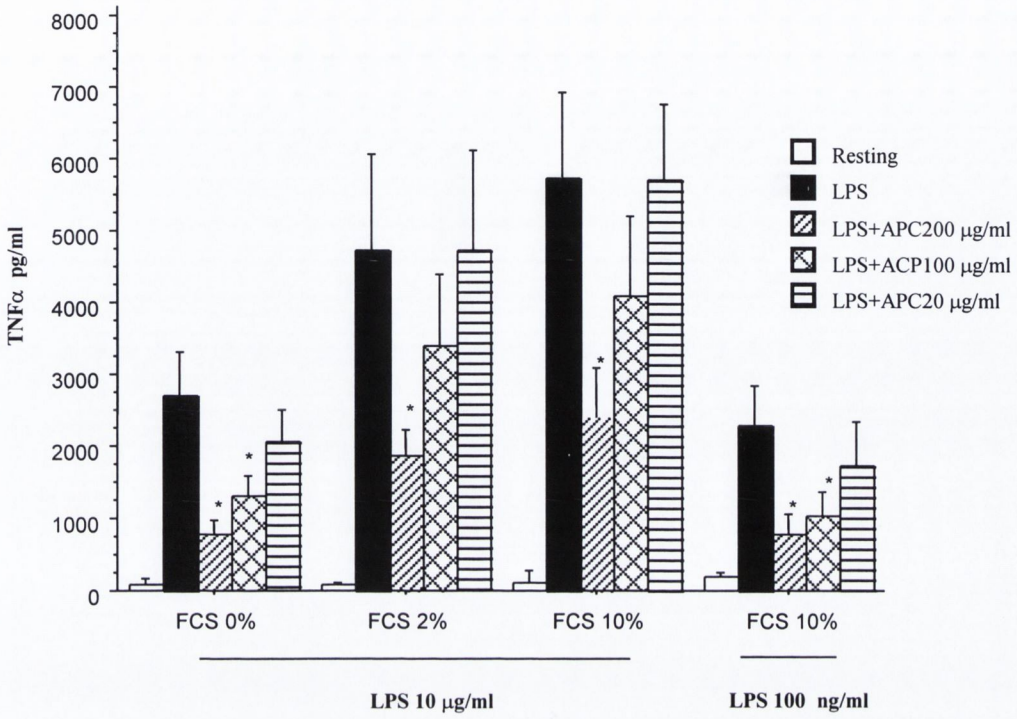
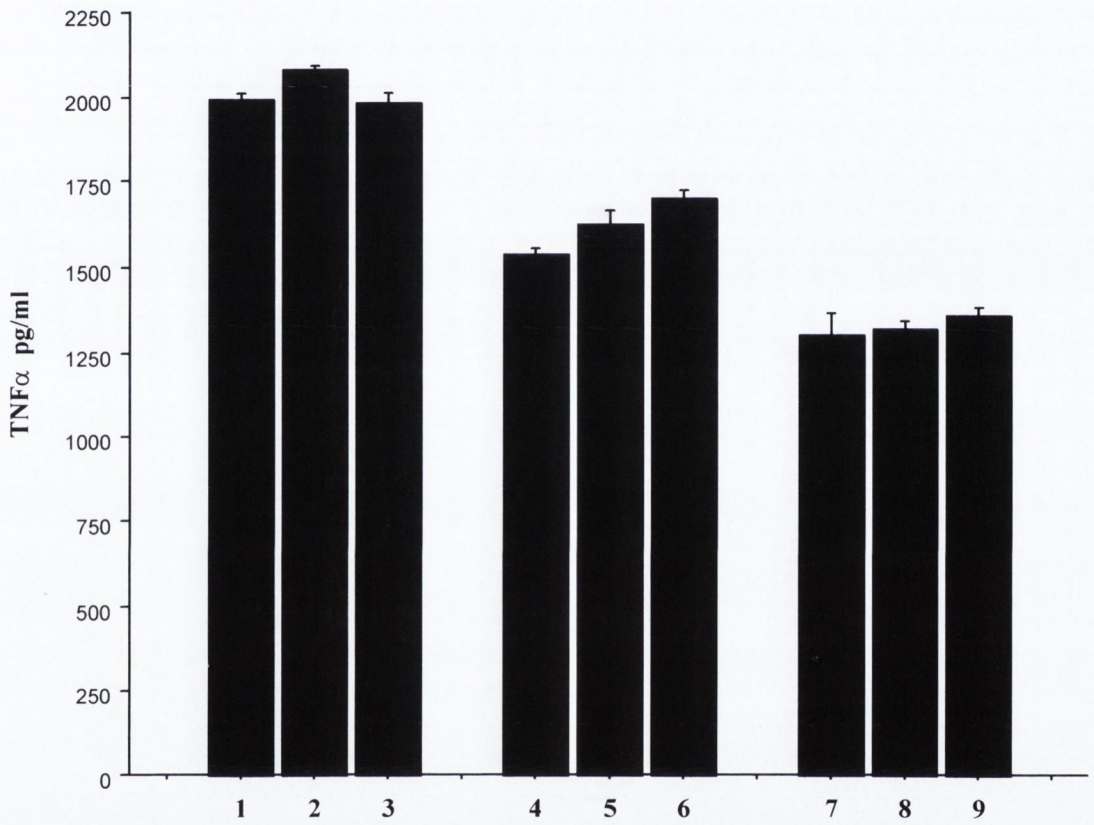


Figure 3.1. Effect of APC on LPS induced TNFα production in THP-1 cell line.

THP-1 cells ($2x10^9$/ml) were preincubated with APC at varying concentrations (200μg/ml, 100μg/ml and 20μg/ml) prior to the addition of LPS 100ng/ml and 10μg/ml. The effect of fetal calf serum (FCS) was assessed by varying the concentration of FCS (0%, 2% and 10%) in the cell culture medium immediately prior to the addition of LPS 10μg/ml. The concentration of TNFα in the supernatant was determined at four hours by ELISA. FCS resulted in a dose dependant increase in LPS induced TNFα production ($p<0.001$ by analysis of variance (Anova)). APC resulted in significant inhibition of TNFα production. The most marked inhibition was seen at a concentration APC of 200μg/ml, which blocked the effect of 100 ng/ml LPS by 75.3+/-2.9%. The inhibitory effects of APC were less marked at the higher concentration of LPS (10μg/ml) and in the presence of FCS, however APC 200μg/ml resulted in at least a 50% reduction in TNFα under all conditions tested. Data is expressed as mean +/-1 SD of triplicate experiments. The percentage inhibition of TNFα at different concentrations was analysed by the Mann Whitney test, *$p<0.05$.



1 = TNFα 2000pg/ml , **2** = TNFα 2000pg/ml + APC 200μg/ml, **3** = TNFα 2000pg/ml + PC 200μg/ml

4 = TNFα 1550pg/ml , **5** = TNFα 1550pg/ml + APC 200μg/ml, **6** = TNFα 1550pg/ml + PC 200μg/ml

7 = TNFα 1300pg/ml , **8** = TNFα 1300pg/ml + APC 200μg/ml, **9** = TNFα 1300pg/ml + PC 200μg/ml

Figure 3.2. Effect of APC and PC on TNFα standards.

Varying concentrations of TNFα standards (2000pg/ml, 1550pg/ml, and 1300pg/ml) were incubated alone or with APC (200μg/ml) or with PC (200μg/ml). The concentration of TNFα was measured at four hours by ELISA. Data is expressed as mean +/-1 SD of triplicate experiments.

Discussion

This study has demonstrated that APC inhibits LPS induced TNFα production in a THP-1 cell line. The inhibitory effect did not result from direct inactivation of TNFα in the supernatant, since the incubation of TNFα standards with APC 200μg/ml for four hours had no effect on TNFα activity. The ability of APC to inhibit TNFα is likely to be significant given the overwhelming experimental evidence to suggest that this pro-inflammatory cytokine plays a pivotal role in endotoxin induced multiorgan failure (Beutler *et al*, 1985; Chollet-Martin *et al*, 1992). The exposure of monocytes to LPS, *in vitro,* results in increased TNFα production. Serum TNFα concentrations are increased after infusion of *E. coli* or LPS and the circulating level of TNFα is predictive of clinical outcome in patients with septic shock (Waage *et al*, 1987; Waage & Steinshamn, 1993). Furthermore, many of the biological effects of TNFα infusions are similar to those observed during a septic event. Infusion of TNFα, in humans, result in the dose dependant development of fever, malaise, headache, hypotension, organ dysfunction, activation of the coagulation cascade and elevation in the peripheral blood neutrophil count (Schiller *et al*, 1991; Gamm *et al*, 1991; Jones & Selby, 1989). The importance of TNFα in the pathogenesis of septic shock is supported by the demonstration that neutralising antibodies to TNFα reduced the mortality associated with lethal doses of *E. coli* in mice and subsequent studies showed similar results in primate models of sepsis (Bodmer *et al*, 1993). However, recent randomised clinical trials, assessing the efficacy of monoclonal anti-TNFα antibodies in sepsis, have yielded disappointing results with no apparent benefit in the treatment arm (Abraham *et al*, 1995). The results of these clinical trials suggest that either TNFα is not important in

the pathophysiolgy of sepsis or, perhaps more likely, that inhibition of a specific cytokine such as TNFα may be insufficient to reverse a process that at the time of anti-cytokine therapy involves a wide variety of pro-inflammatory molecules with overlapping biological functions. The ability of APC to inhibit IL-1β, in addition to TNFα, suggest that it has a more wide ranging immunomodulatory properties than anti-TNFα therapy and that PC replacement may represent a more effective treatment strategy for sepsis syndromes. Furthermore, APCD deficiency may occur very early in the evolution of the inflammatory response, at a time when TNFα is the primary cytokine.

The presence of FCS resulted in a dose dependent increase in LPS induced TNFα production, and a reduction in the inhibitory effects of APC. The increase in TNFα associated with FCS likely reflects the presence of co-stimulatory molecules such as LPS binding proteins in the serum (Fenton & Golenbock, 1998). The reduction in the inhibitory effect of APC, in the presence of FCS, may reflect the co-stimulatory effect of the later or may result from the presence of physiological inhibitors of APC such as α1-antitrypsin, α2-macroglobulin or PC inhibitor, at normal or increased concentrations in FCS (Heeb *et al*, 1991; Suzuki *et al*, 1989; Suzuki *et al*, 1989). Therefore, the protective effect of APC in sepsis may be influenced by the concentration of LPS, the circulating levels of APC/PC, and the concentration of LPS co-stimulatory factors or PC inhibitors in the serum.

In summary APC is capable of inhibiting LPS induced TNFα production and this effect is influenced by the concentration of FCS in the culture medium. The ability of APC to inhibit TNFα identifies a potentially important pathway in the pathophysiology and treatment of multiorgan failure associated with Gram negative sepsis. Further work is required to define the cellular mechanism(s) by which APC inhibits TNFα production.

Chapter 4

Introduction

The activation of monocytes by LPS leads to the initiation of proximal signalling events, which result in the production of TNFα. This effect appears to be mediated by an increase in messenger RNA (mRNA), an increase in translational efficiency and the release of cytoplasmic stores of TNFα (Yao *et al,* 1997; Zuckerman *et al,* 1991; Han *et al,* 1990). The ability of APC to decrease LPS induced TNFα production suggests that it effects one of these cellular mechanisms. We assessed the effect of APC and PC on LPS induced increase in TNFα mRNA.

Materials and methods:

Activated Protein C

Activated PC (lot PCA 162) was provided by Baxter Hyland Immuno, Vienna, Austria. Protein C was isolated from human plasma by immunoaffinity chromatography with anti-PC monoclonal antibody. Activated PC was prepared by activation of immunopurified PC with human thrombin followed by separation by ion exchange chromatography and was certified as sterile and pyrogen free by the manufacturer.

Cell culture

The human monocyte THP-1 cell line (European collection of cell cultures, Salisbury, UK) was cultured at 37°C in 5% CO_2 at a density of 2-9x10^5 per ml. The cell culture medium was RPMI-1640 (Gibco BRL, UK) supplemented with 2mM glutamine (Sigma, UK), 10% fetal calf serum (FCS) (Gibco BRL, UK), penicillin 50units/ml and of streptomycin 50units/ml (Gibco BRL, UK). Cells were counted and >90% viability was confirmed by staining with ethidium bromide acridine orange (Sigma, UK). The cells were then resuspended at a concentration of 2x10^6/ml in culture medium, which did not contain FCS. A 2ml cell suspension was transferred to a six well plate and incubated with APC 200μg/ml or PC 200μg/ml ten minutes prior to addition of LPS (*E coli* 0127, Sigma, UK) at a concentration 10μg/ml. The reaction was stopped after two hours by the addition of 2mls of ice cold phosphate buffered saline (PBS) (Gibco BRL, UK). Following centrifugation at 1000 g/min the supernatant was collected and stored at -70°C for subsequent measurement of TNFα by ELISA (see chapter 3). The concentration of TNFα mRNA was measured in the cells.

Overview of the method used in the measurement of TNF mRNA

TNFα mRNA was measured by reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was extracted from THP-1 cells and reverse transcribed to cDNA. The cDNA was amplified in the presence of a varying concentrations of a competitor. The band intensities of TNFα and competitor were measured by densitometry. The concentration of TNFα mRNA was calculated on the basis that it was equivalent to that concentration of competitor, which resulted in amplification products of similar band intensity. The concentration of TNFα mRNA was corrected for Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (figure 4.1).

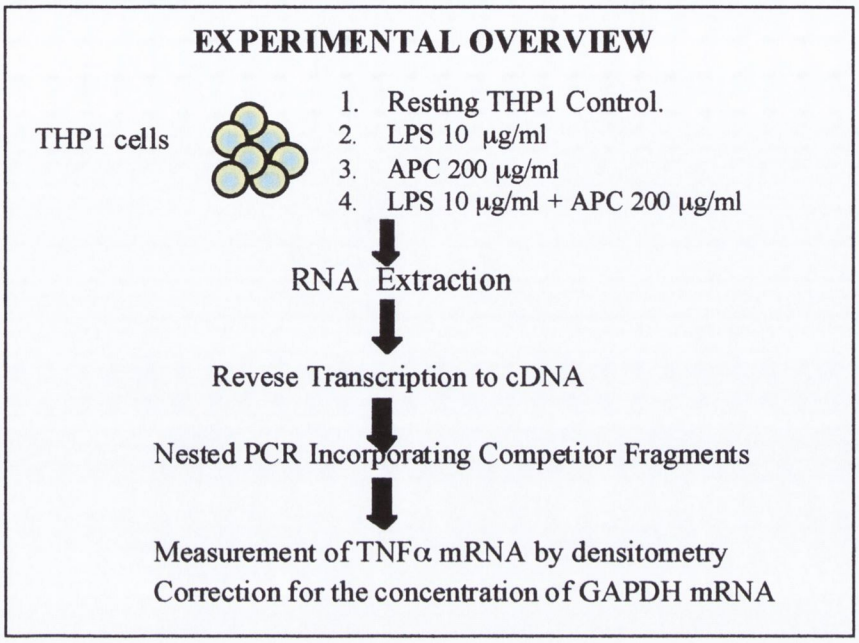


Figure 4.1. General overview of methodology used to quantify TNFα mRNA

Primer design

Nested primers were designed for TNFα cDNA (Genosys-Sigma, UK) (figure 4.2 and table 4.1). The inner sense and outer antisense primers spanned the exon-intron junction and thereby prevented the amplification of genomic DNA. A single set of primers were designed for GAPDH cDNA, which did not span the exon-intron junction and were capable of amplifying residual genomic DNA, resulting in a distinct amplification product which includes intronic sequences. This acts as an internal control to detect the presence of genomic DNA, which would otherwise compete with cDNA and reduce amplification efficiency.

Competitor Construction

The competitor was generated from Lambda DNA (Boehringer-Mannheim Roche, UK) (Figure 4.3 and Table 4.1). Lambda is a bacteriophage and shares minimal sequence homology with genomic DNA. Inner sense and inner antisense hybrid primers were designed so that their 3' ends were complimentary to the Lambda DNA. The outer ends of these primers were tagged with sequences from TNFα inner sense and inner antisense primers so that the amplified primary product included Lambda DNA in addition to TNFα sequences at the 5' and 3'ends. Outer sense and outer antisense hybrid primers were then designed so that their 3' ends were complimentary to the amplified primary product. The outer ends of these primers were tagged with sequences from TNFα outer sense and outer antisense primers. The final product contained an inner Lambda DNA with TNF sequences at either end, which were complimentary to the both the inner and outer set of TNFα primers (Figure 4.3). When amplified, using

TNFα primers, the competitor amplicon is longer than the TNFα product and therefore can be distinguished by electrophoresis. The length of the competitor amplicon was determined by the distance between the inner primers on the lambda DNA. The internal Lambda sequence is sufficiently different from TNFα to prevent heteroduplex formation between competitor and TNFα, which would otherwise reduce amplification efficiency.

The PCR reaction was performed in a final volume of 50μl, containing 0.4 units of DNA Taq polymerase, 0.25μg of lamda DNA (Boehringer-Mannheim Roche, UK), 50mM KCl, 10mM Tris-HCl pH 9.0, Triton X-100, 1.5mM $MgCl_2$, 4% dimethyl sulphoxide (BDH, UK), 30mM each of deoxyribonucleoside triphosphates (Boehringer-Mannheim, Roche) and 0.025μM of the inner hybrid primer (table 4.1). Amplification for 7 cycles was performed with the following cycling parameters: denaturation at 94°C for 60 seconds, primer annealing at 55°C for 45 seconds, and an extension step at 72°C for 90 seconds. One μl of the primary product was transferred to a second reaction mixture. The reaction mix and conditions were similar to first round PCR but utilised the outer set of hybrid primers at a concentration of 0.05μM (Table 4.1). In addition, the number of amplification cycles was increased from 7 to 32 cycles. 20μl of the amplified product was separated by electrophoresis on a 3% weight/volume agarose gel (Gibco BRL, UK) containing 10μg/ml ethidium bromide (Sigma, UK) and visualized by UV transillumination. The secondary product was diluted 10,000 fold in nuclease free water (Promega, UK) and re-amplified using the TNFα specific outer primers. The higher concentration of outer hybrid primer and the increase in the number of amplification

cycles favoured the second PCR reaction and therefore limited the concentration of the primary competitor product (i.e. lambda DNA with inner TNFα sequences only) which would have competed for amplification with the final product. The ratio of final competitor product to the primary product was further minimised by repeating the secondary PCR reaction. A competitor for GAPDH was generated in a similar manner using single set of primers with one round of amplification.

2μl of the final competitor products was added to 40mls of nuclease free water. Tenfold dilutions were prepared by adding 2mls of each dilution to 18mls of nuclease free water. The concentration of competitor was calculated from the mean of triplicate spectrophotometry readings at 260nm and the dilutions were stored at –20°C in 200μl aliquots.

μg/ml of competitor = optical density at wavelength 260 nm x dilution factor x 50 optical density units.

Primer Code	Sequence
TNF-Competitor Outer sense	5'-TGAGCACTGAAAGCATGATCACGTGGAGCTGGCCGAGGAG-3'
TNF-Competitor Outer antisense	5'-GTTTGCTACAACATGGGCTAGAAGATGATCTGACTGCCTG-3'
TNF-Competitor Inner sense	5'-ACGTGGAGCTGGCCGAGGAGGTGCGCTGGCGTATGCCTG-3'
TNF-Competitor Inner antisense	5'-GAAGATGATCTGACTGCCTGGCCTGATTGCCGGAAAGGAC-3'
GAPDH-Competitor-Sense	5'-GAAGGTGAAGGTCGGAGTCGTGCGCTGGCGTATGCCTG-3'
GAPDH-Competitor-Antisense	5'-GAAGATGGTGATGGGATTTCGCCTGATTGCCGGAAAGGAC-3'
TNF-Outer sense	5'-TGAGCACTGAAAGCATGATC-3'
TNF- Outer antisense	5'-GTTTGCTACAACATGGGCTA-3'
TNF- Inner sense	5'-ACGTGGAGCTGGCCGAGGAG-3'
TNF- Inner antisense	5'-GAAGATGATCTGACTGCCTG-3'
GAPDH-Sense	5'-GGAAGGTGAAGGTCGGAGTC-3'
GAPDH-Antisense	5'-GAAGATGGTGATGGGATTTC-3'

Table 4.1 Primers used for competitor generation and for amplification of TNFα and GAPDH mRNA. This table illustrates the primers, which were used for the amplification of TNFα and GAPDH mRNA. Hybrid primers were only used for the generation of the competitor sequences and are designated as 'TNF-competitor' or 'GAPDH-competitor'.

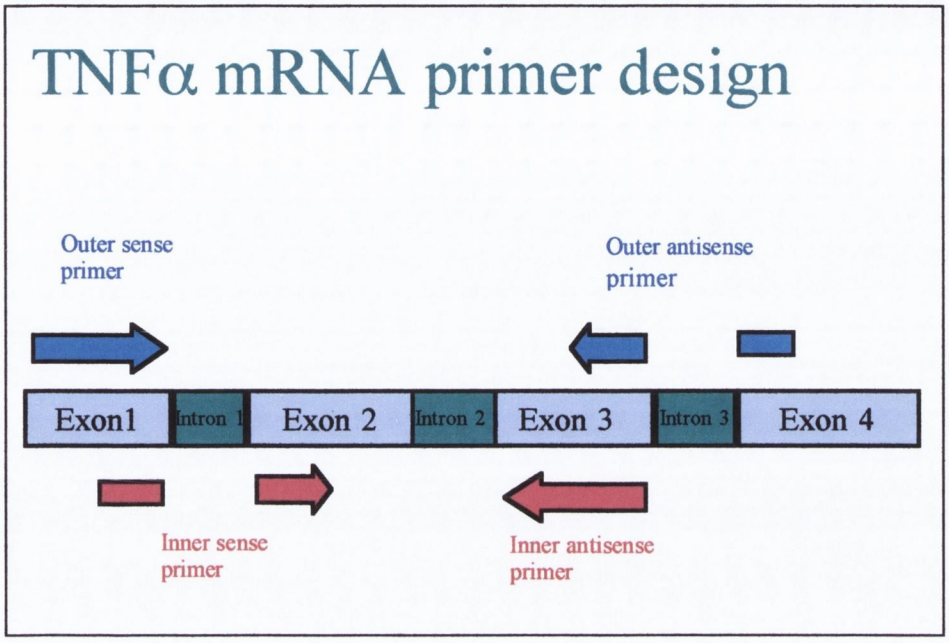


Figure 4.2. Primer design for TNFα mRNA.

Nested primers were designed to TNFα RNA. These primers spanned the intron-exon boundaries and therefore prevented the amplification of genomic DNA.

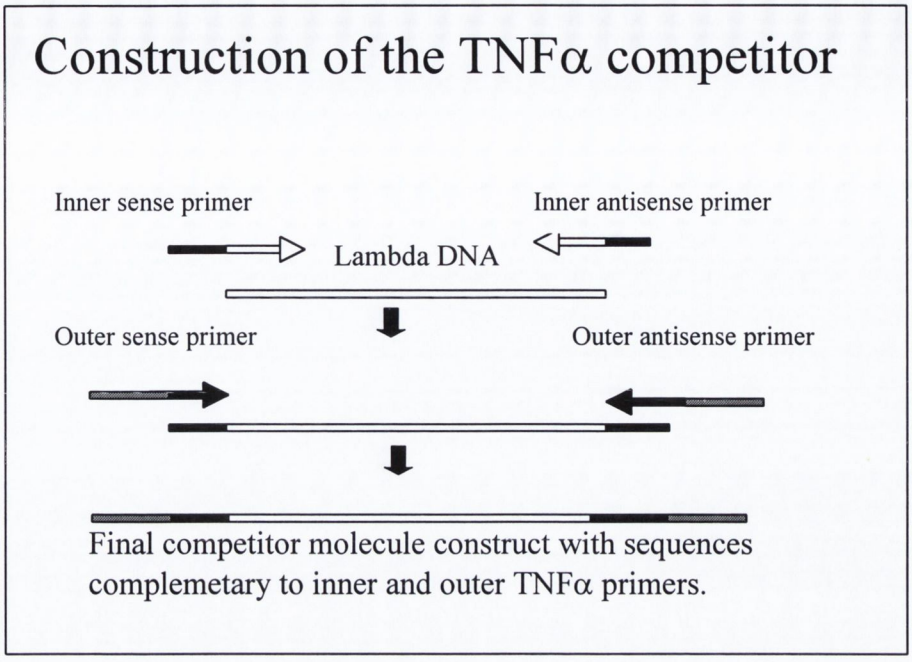


Figure 4.3 Overview of competitor construction

Competitors were constructed for GAPDH and TNFα mRNA by PCR amplification of Lambda DNA with the hybrid primers (see Table 4.1):

RNA Extraction

RNA was isolated directly from the semi-adherent cells using a combination of two commercial kits, (RNA extraction kit, Flowgen PURE*script*, UK and DNA Shredder Columns, Quiagen, UK), according to the manufacturer's instructions (table 4.2). Briefly, the supernatant and PBS (Gibco BRL, UK) was removed from the cell culture plates by centrifugation. Cell lysis solution (PURE*script*, UK) was added and the lysate was spun over a Quiagen shredder column in order to shear any genomic DNA. Protein and DNA were initially removed by percipitation and RNA was subsequently precipitated from the supernatant using solutions provided by the manufacturer (PURE*script*, UK). Complimentary DNA was synthesized immediately after RNA extraction in order to limit RNA degradation.

cDNA Synthesis

Complimentary DNA was synthesized using 0.5units AMV (Avian Myeloblastosis Virus) reverse transcriptase enzyme (Promega, UK), 1X Reverse Transcriptase buffer (50mM Tris HCL pH 8.3, 50mM $MgCl_2$, 5 mM DTT, 5mM EDTA (BDH, UK), 50μg/μl BSA, and 50mM KCL), 0.8mM deoxyribonucleoside triphosphates (Boehringer-Mannheim, Roche, UK), 10% dimethyl sulphoxide (DMSO), 0.75units RNAsin (PURE*script*, UK), 0.1μM of each outer antisense primer (GAPDH and TNFα) and 5μl of RNA in a final volume of 30μl. The reaction mix was incubated at 42°C for 1 hour and then stored at –20°C.

	$1X10^6$	$2x10^6$	$3x10^6$	$10x10^6$
CELL LYSIS				
PBS	10µl	20µl	30µl	100µl
Lysis solution	300µl	300µl	400µl	3mls
PROTEIN-DNA PRECIPITATION				
Precipitation sln.	100µl	100µl	200µl	1ml
Inversion X	50	50	50	50
Ice Bath	5 minutes	5 minutes	6 minutes	10 minutes
Centrifuge time	3 minutes	3 minutes	3.5 minutes	5 minutes
RNA PRECIPITATION				
Isopropanol	300µl	300µl	400µl	3mls
Centrifuge time	3 minutes	3 minutes	3.5 minutes	5 minutes
70% ethanol	300µl	300µl	400µl	3mls
RNA HYDRATION				
RNA hydration Sln	50µl	50µl	60µl	20µl

Table 4.2. Reaction mixture for RNA extraction

RNA was extracted from cells using a commercial RNA extraction kit (RNA extraction kit, Flowgen PURE*script*, UK and DNA Shredder Columns, Quiagen, UK) according to the manufacturer's instructions. Cell lysis solution, precipitation solution and isopropanol were provided by the manufacturer. "Inversion X" refers to the number of times the reaction mixture was inverted. Centrifugation was performed at 19,000g in a minicentrifuge.

Reagent	Stock concentration	Volume/ Reaction	Volume/10 reactions
RT Buffer	5X	6 µl	60µl
dNTPs	4 mM	4.5µl	45µl
DMSO	100%	4.5µl	45µl
Outer antisense GAPDH primer	100 pmol	1.5µl	15µl
Outer antisense TNFα primer	100 pmol	1.5µl	15µl
Nuclease Free water		To 22.5µl	To 225 µl
Rnasin	5-10 U/µl	0.5µl	5 µl
RT Enzyme	5-10 U/µl	0.5µl	5 µl

RT= reverse transcriptase

Table 4.3 Reaction mixture for reverse transcription of TNFα and GAPDH mRNA.

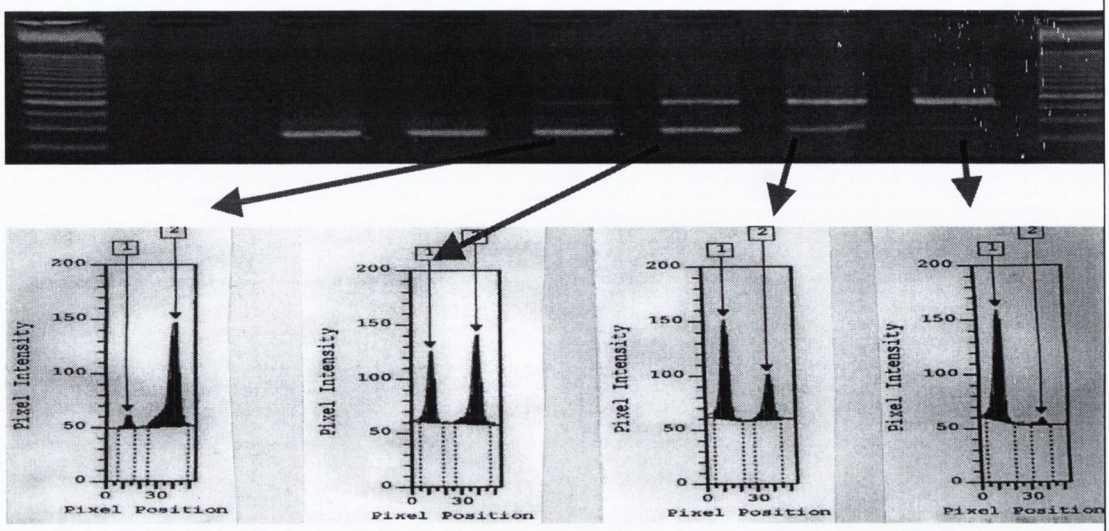
TNFα and GAPDH cDNA were synthesized from total RNA using 0.5units of the reverse transcriptase enzyme AMV (Avian Myeloblastosis Virus) (Promega, UK), and the outer antisense primers for TNFα and GAPDH. The concentrations and volume of the components of the reaction mixture are illustrated.

Competitive PCR for TNFα

The competitive PCR reaction for TNFα cDNA involves two sequential PCR reactions using two sets of primers (outer and inner) and allows the amplification from a single copy of TNFα cDNA (Figure 4.2) (Simmonds *et al,* 1990). Eight PCR reactions were required to measure TNFα cDNA in each sample. One tube contained competitor only, one contained the sample TNFα cDNA only and the remaining six tubes contained the TNFα cDNA with log fold dilutions of competitor ($1.72x10^{5}$ – $1.72x10^{-1}$ fg). Each first round PCR reaction was performed in a final volume of 50μl, containing 0.4units of DNA Taq polymerase, 2μl of cDNA, 2μl of competitor DNA, 50mM KCl, 10mM Tris-HCl pH 9.0, Triton X-100, 1.5mM $MgCl_2$, 4% dimethyl sulphoxide (BDH, UK), 30mM each of deoxyribonucleoside triphosphates (Boehringer-Mannheim, Roche, UK) and 0.025μM of TNFα outer-sense and TNFα outer-antisense primer (table 4.1). Amplification for 15 cycles was performed with the following cycling parameters: denaturation at 94°C for 60 seconds, primer annealing at 55°C for 45 seconds, and an extension step at 72°C for 90 seconds. One μl of the primary product was transferred to a second reaction mixture. The reaction mix and conditions were similar to first round PCR but a second "nested" set of primers, situated within the primary set were used (table 4.1, figure 4.2). The primer concentration was increased to 0.05μM and the number of amplification cycles was increased from 15 to 32 cycles. 20μl of the amplified product was separated by electrophoresis on a 3% weight/volume agarose gel (Gibco BRL, UK) containing 10μg/ml ethidium bromide (Sigma, UK) and visualized by UV transillumination

Competitive PCR for GAPDH

The concentration of GAPDH, a 'housekeeping' gene, was measured using a single set of primers (table 4.1). Eight PCR reactions were required to measure the amount of GAPDH cDNA in one sample. One tube contained competitor only, one contained the sample GAPDH cDNA only and the remaining six tubes contained the GAPDH cDNA with log fold dilutions of competitor ($2.8x10^{5}$–$2.8x10^{-1}$ fg). Each PCR reaction was performed in a 50µl volume containing 0.4units of DNA Taq polymerase, 2µl of cDNA, 2µl of competitor DNA, 50mM KCl, 10mM Tris-HCl pH 9.0, Triton X-100, 1.5mM $MgCl_2$, 4% dimethyl sulphoxide (DMSO) (BDH, UK), 30mM each of deoxyribonucleoside triphosphates (Boehringer-Mannheim, Roche), and 0.05µM of GAPDH sense and GAPDH antisense primer. Amplification for 32 cycles was performed with the following cycling parameters: denaturation at 94°C for 60 seconds, primer annealing at 55°C for 45 seconds, and an extension step at 72°C for 90 seconds. 20µl of the amplified product was separated by electrophoresis on a 3% weight/volume agarose gel (Gibco BRL, UK) containing 10µg/ml ethidium bromide (Sigma, UK) and visualized by UV transillumination.



Band	Lane 1	Lane 2	Lane 3	Lane 4
TNFα	3,639	21,559	34,368	43,116
Competitor	48,584	34,701	17,850	2,390

Figure 4.4 Agarose gel electrophoresis and analysis by densitometry of competitor and TNFα mRNA.

The amplification products of competitive PCR for TNFα were separated by electrophoresis on a 3% weight/volume agarose gel containing 10μg/ml ethidium bromide and visualized by UV transillumination. The lower band represents the competitor. The intensities of the competitor and TNFα bands were quantified using an Ultraviolet Transilluminator (UVP$^{TM)}$ and Grab-IT^{TM} (Synoptics) and Gelworks 1D Advanced^{TM} (NonLinear Dynamics Ltd) software.

Quantification of mRNA

The intensities of the competitor and TNF-α bands were quantified using an Ultraviolet Transilluminator (UVP$^{TM)}$ and Grab-IT^{TM} (Synoptics) and Gelworks 1D Advanced^{TM} (NonLinear Dynamics Ltd) software. The concentration of TNFα mRNA is equivalent to the concentration of competitor, added to the PCR reaction, which results in TNFα and competitor bands of similar intensity. This is called the equivalence point. The concentration of competitor at the equivalence point was calculated by simple regression analysis. The ratios of the TNFα/Competitor band intensities (y-axis) were log transformed and plotted against the log transformed concentrations of the competitor (x-axis) using Excel software, Microsoft™ (Figure 4.4). The concentration of TNFα mRNA at the equivalence corresponds to the intersection of the line with the x axis and can be calculated form the equation of the line (Connolly *et al,* 1995). The concentration of TNFα mRNA was corrected for GAPDH mRNA.

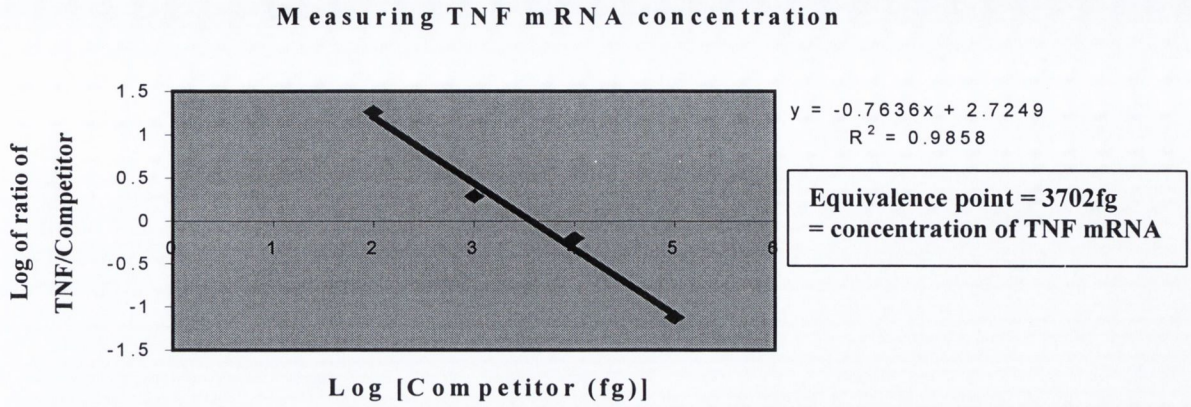


Figure 4.5 Calculation of the concentration of TNF mRNA.

The log of [TNFα:Competitor] band intensities (y-axis) were graphed against the log of the concentrations of competitor (x-axis). The equation of this line was calculated. At the equivalence point, TNFα = competitor, [TNFα/Competitor] =1, log [TNFα/Competitor] = 0, and y=0. The value of x (competitor concentration) when y = 0 was calculated from the equation of the line and is equivalent to the concentration of TNFα mRNA in the sample. This value can be visualised on the graph, at the intersection of the line with the x axis.

Results

LPS at 10μg/ml was a strong stimulator of THP-1 cells, and resulted in a 13 +/- 7.4 fold (mean +/- sd) increase in TNFα mRNA (figure 4.8). The maximum effect occured two hours after the addition of LPS (figure 4.6). Pre-incubation with APC (200μg/ml) completely inhibited the LPS induced increase in TNFα mRNA ($p<0.05$) (figure 4.7 and 4.8). This was associated with a significant reduction in TNFα production in the supernatant (figure 4.9). The addition of APC 200μg/ml to resting cells resulted in a reduction in baseline TNFα mRNA although this effect failed to achieve statistical significance (figure 4.7 and 4.8). Pre-incubation with PC (200μg/ml) also inhibited the LPS induced increase in TNFα, at both protein and mRNA level, although the later failed to achieve statistical significance (figure 4.8 and 4.9).

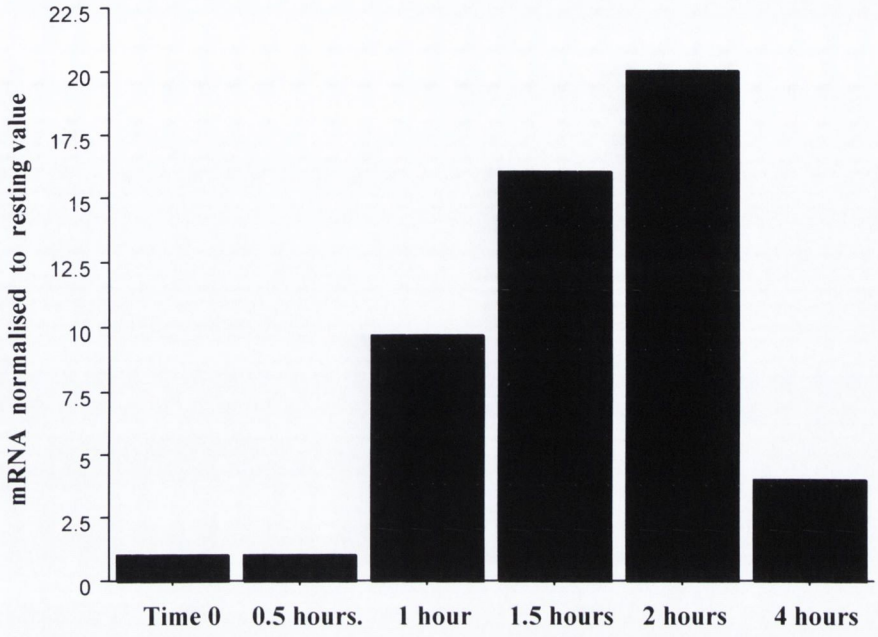


Figure 4.6 Time course for LPS induced increase in TNFα mRNA.

THP-1 cells were exposed to LPS 10μg/ml and the reaction was stopped at 0.5, 1, 1.5, 2, and 4 hours. RNA was isolated form the cells and TNFα mRNA was quantified by a nested competitive reverse transcriptase PCR and visualised by electrophoresis on 3% agarose gel stained with ethidium bromide. The gels were scanned by densitometry and the equivalence point was calculated from the equation of the line where the y-axis represented log [TNFα: competitor] and the x-axis the log [competitor]. The concentration of TNFα mRNA was corrected for GAPDH mRNA and the results were normalised to the value obtained with resting cells. LPS resulted in an increase in TNFα mRNA, which peaked at 2 hours. A representative experiment is presented.

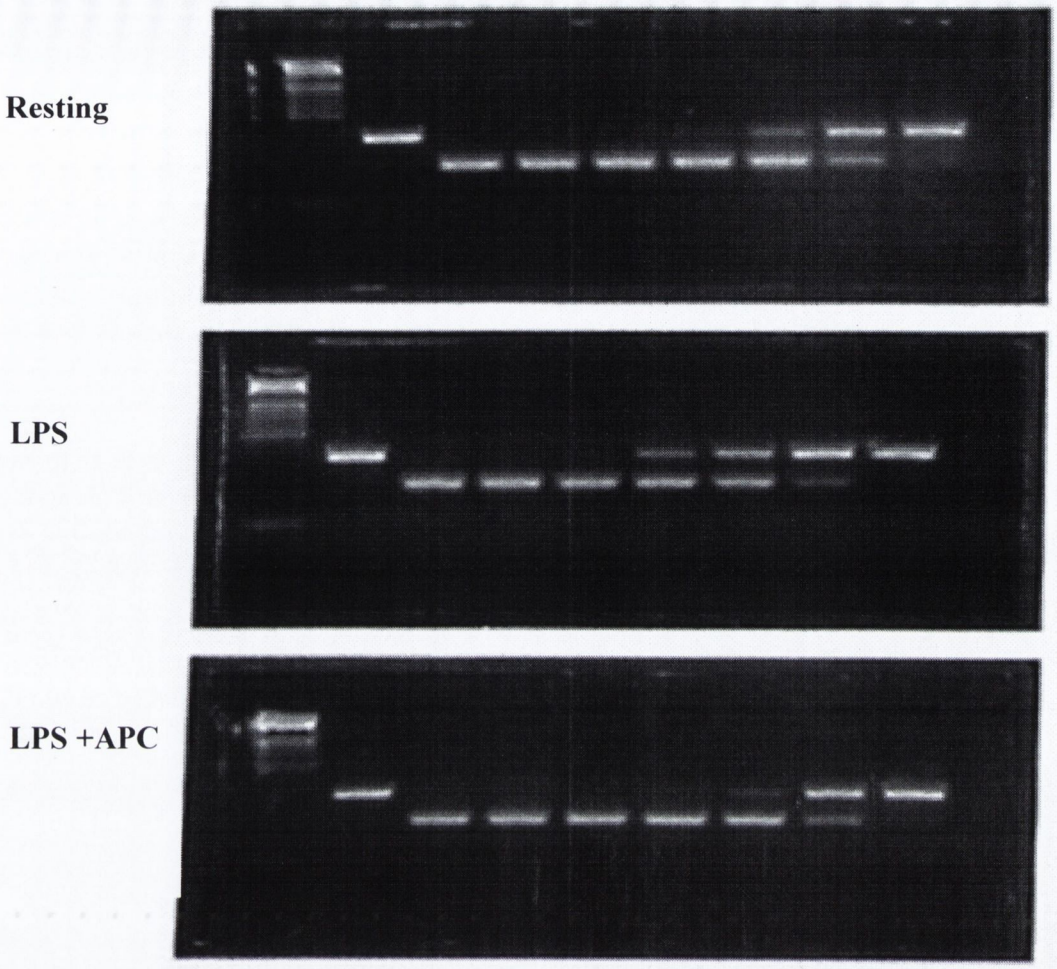


Figure 4.7 APC inhibits LPS induced TNFα mRNA.

THP-1 cells were exposed to LPS 10μg/ml, APC 200μg/ml, LPS 10μg/ml + APC 200μg/ml and LPS 10μg/ml + PC 200μg/ml. RNA was extracted from the cells at two hours and the concentration of TNFα mRNA was assessed by competitive reverse transcriptase PCR. The PCR products were visualised by electrophoresis on 3% agarose gel stained with ethidium bromide. The lower band represents the competitor. The concentration of competitor in each lane decreases by tenfold from right to left. The equivalence point occurs when the band intensity of competitor and TNF are approximately equal. Lane 1= molecular weight marker, lane 2 = TNFα cDNA only, lane 3= competitor only, and lane 4-9 TNFα cDNA with varying concentrations of competitor ($1.72x10^{5}$ – $1.72x10^{-1}$ fg). The equivalence point in resting cells was in lane 8. LPS increased TNFα mRNA and shifts the equivalence point to lane 7, which equates to an approximately ten-fold increase in mRNA. APC and PC (not shown) inhibits the LPS induced increase in TNFα mRNA.

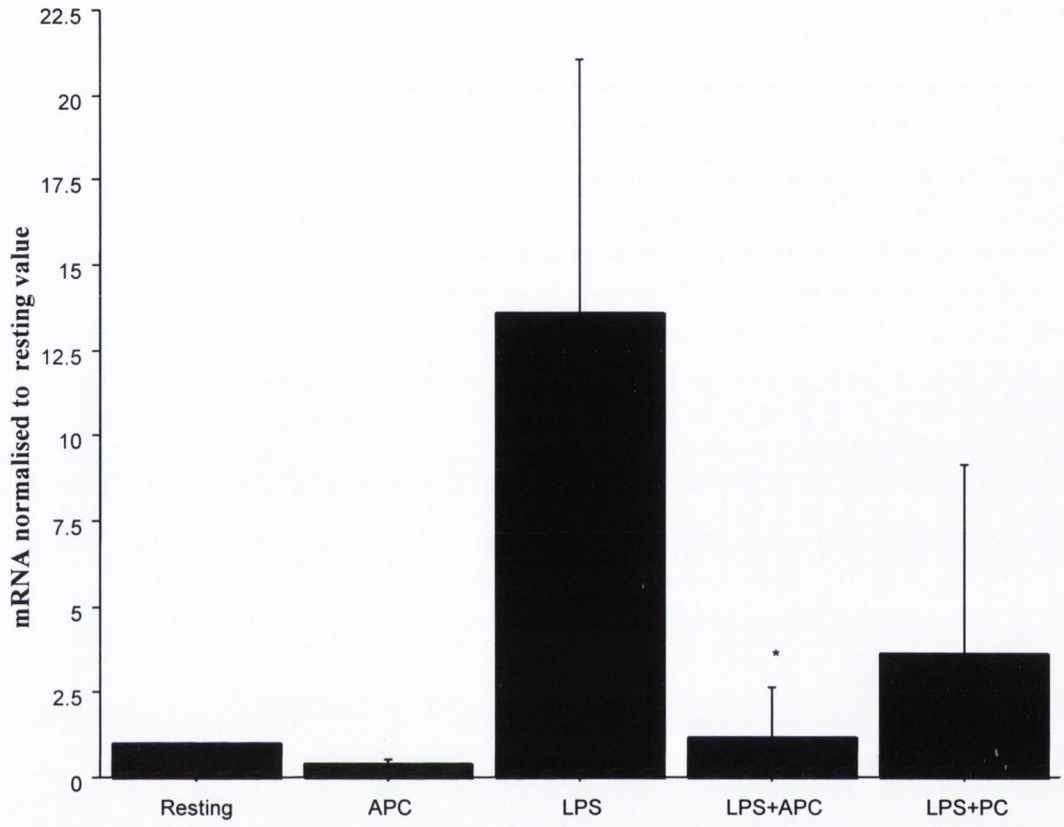


Figure 4.8 APC/PC inhibits LPS induced increase in TNFα mRNA.

The agarose gels representing amplified products of reverse transcription PCR for TNFα and GAPDH mRNA were scanned by densitometry and the concentration of competitor at the equivalence point was calculated from the equation of the line where the y-axis represented log [TNFα: competitor] and the x-axis the log [competitor]. The concentration of TNFα mRNA was corrected for the concentration of GAPDH mRNA and the results were normalised to the value obtained with resting cells. LPS at 10μg/ml resulted in a 13 +/- 7.4 fold (mean +/- sd) increase in TNFα mRNA. Pre incubation with APC 200μg/ml completely inhibited the increase in TNFα mRNA. PC 200μg/ml also inhibited TNFα mRNA although this failed to achieve statistical significance. Data is expressed as the mean +/- 1 SD for triplicate experiments. The Mann Whitney U test was used to compare the TNFα mRNA concentration in cells exposed to LPS alone versus LPS + APC and LPS alone versus LPS + PC, *$p<0.05$.

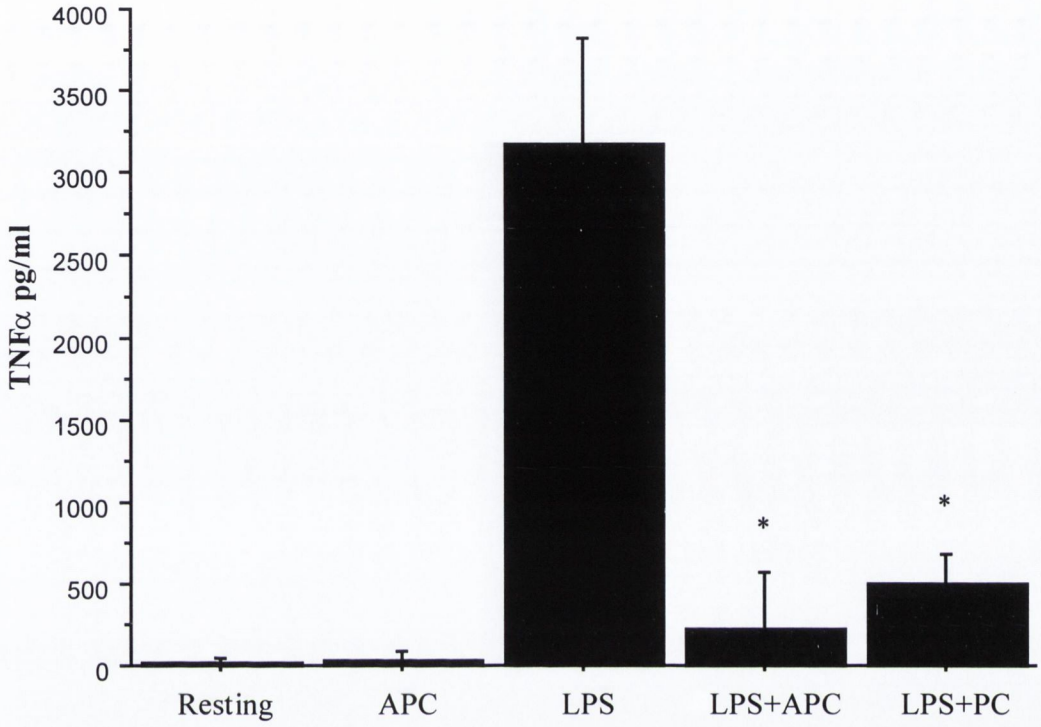


Figure 4.9 APC/PC inhibits LPS induced increase in TNFα in supernatant

THP-1 cells were exposed to LPS 10μg/ml, APC 200μg/ml, LPS 10μg/ml + APC 200μg/ml and LPS 10μg/ml + PC 200μg/ml. The reaction was stopped at two hours and the supernatant separated form the cells. RNA was isolated form the cells and the TNFα concentration in the supernatant was measured by ELISA. APC and PC resulted in significant inhibition of LPS induced increase in TNFα production. Data is expressed as the mean +/- 1 SD for triplicate experiments. The Mann Whitney U test was used to compare the TNFα concentration in cells exposed to LPS alone versus LPS + APC and LPS alone versus LPS + PC, *$p<0.05$.

Discussion

A competitive reverse transcriptase PCR (RT-PCR) assay, corrected for GAPDH, was used to quantify TNFα mRNA expression. The use of a competitor improved the accuracy of the mRNA assay and it also corrected for differences in PCR amplification efficiency, since any variations would affect TNFα and competitor equally. Furthermore, the measurement of GAPDH mRNA allowed for the correction of variations due to differences in cell number, RNA extraction efficiency, and RNA degradation. The incorporation of nested primers for TNFα amplification improved both the sensitivity and specificity of the assay (Simmonds *et al,* 1990). The increased sensitivity was associated with an increased risk of contamination, however, this was prevented by spatial separation of the experiments and the use separate equipment for RNA extraction, buffer preparation, reverse transcription and PCR amplification.

Non-exponential accumulation of PCR product or the 'plateau effect' occurs when the PCR reaction is continued beyond a certain point. When this occurs, it is not possible to accurately measure target cDNA since significantly different starting concentrations can lead to similar amounts of final product. The risk of developing a plateau effect was highest in the first PCR reaction because the template and primers would be exposed to both the first and second round PCR. This effect was reduced by generating conditions which favoured the second PCR reaction and involved increasing the number of amplification cycles and the primer concentration.

Protein C demonstrated similar anti-inflammatory properties to APC. This finding is at variance with previously published data from another group (Grey *et al*, 1993). In both studies the cell line, type of LPS and TNFα assay were similar. We used a commercially available PC concentrate (Hyland-Baxter) which was purified by monoclonal antibodies and inactivated by a solvent detergent and vapour heated method. Grey *et al,* purified PC from plasma collected from blood donors although the precise details of purification process were not defined. It is possible that the discrepant results represent differences in the isolation of PC from plasma or the viral inactivation process. The cause of the discrepant results requires further study.

The mechanism responsible for the LPS induced increase in TNFα mRNA involves an increase in both stability and transcription. The increase in stability appears to be mediated at least in part by regulatory sequences within the 3'untranslated region of the TNFα gene (Zuckerman *et al*, 1991). Increased transcription requires the concerted binding of NFκB, Egr-1 and c-Jun to the promoter region of the TNFα gene (Yao *et al*, 1997). The importance of NFκB in enhancing transcription is supported by studies, in which transfection with TNFα promoter constructs lacking the κB3 binding site for NFκB, resulted in a significant reduction in LPS induced transcription of the downstream reporter gene (Yao *et al*, 1997). We have demonstrated that APC inhibits the LPS induced increase in TNFα mRNA. Further studies are required to determine the molecular mechanism(s) by which APC mediates this effect. The pivotal role of NFκB in the generation of a wide variety of pro-inflammatory molecules including TNFα

identifies this transcription factor as a potentially important molecular mechanism by which APC inhibits TNFα mRNA.

Chapter 5

Introduction

NFκB functions as a heterodimer usually consisting of p65 and p50 protein subunits (Siebenlist *et al*, 1994). It is a ubiquitous transcription factor that was originally identified as a regulator of kappa like chain genes in B lymphocytes (Sen & Baltimore, 1986), but has subsequently been shown to regulate the expression of a wide variety of genes involved in the immune and inflammatory response (Kopp & Ghosh, 1995). In unstimulated cells, NFκB is localised within the cytoplasm by binding to IκBα and IκB β, which prevents nuclear translocation (Baldwin, 1996). Activation of cells with LPS, cytokines, viruses, oxidants or activators of protein kinase C leads to the induction of several signal transduction pathways which result in the phosphorylation of IκB kinase (DiDonato *et al*, 1996). The subsequent phosphorylation of IκB results in rapid proteasomal degradation with release of NFκB which in turn is translocated into the nucleus where it binds to specific sequences in the promoter regions of target genes responsible for the generation of pro-inflammatory cytokines, chemokines, enzymes that generate mediators of inflammation, immune receptors and adhesion molecules (Kopp & Ghosh, 1995; Liu *et al*, 1997; DiDonato *et al*, 1996; Barnes & Karin, 1997) (figure 5.1). These are the same inflammatory mediators that result in the development of endotoxin induced multiorgan failure and are responsible for the high predicted mortality associated with septic shock (Moncada & Higgs, 1991; Wolkow, 1998; Waage *et al*, 1991; Halstensen *et al*, 1993; Endo *et al*, 1995; Sessler *et al*, 1995; Cowley *et al*, 1994). The pro-inflammatory cytokines, TNFα and IL-1β, cause further activation of NFκB providing a positive feedback loop (Kopp & Ghosh, 1995). Therefore, the activation of NFκB in response to LPS leads to a co-ordinated increase

in a wide variety of pro-inflammatory molecules which in turn lead to amplification and perpetuation of the inflammatory response (Barnes & Karin, 1997). The importance of NFκB in the systemic inflammatory response syndrome is supported by data which demonstrated that NFκB activity was higher in peripheral blood mononuclear cells from patients who died of sepsis in comparison to those who survived (Bohrer *et al*, 1997). Furthermore, the inhibition of NFκB is associated with a significant reduction in mortality in a murine model of sepsis (Kovacich *et al*, 1999). We identified NFκB as a potential molecular target for APC. It was postulated that the protective effect of APC in sepsis and its ability to downregulate TNFα mRNA was mediated by inhibition NFκB. The ability of APC to inhibit LPS-induced nuclear translocation of NFκB was assessed in a THP-1 monocyte cell line.

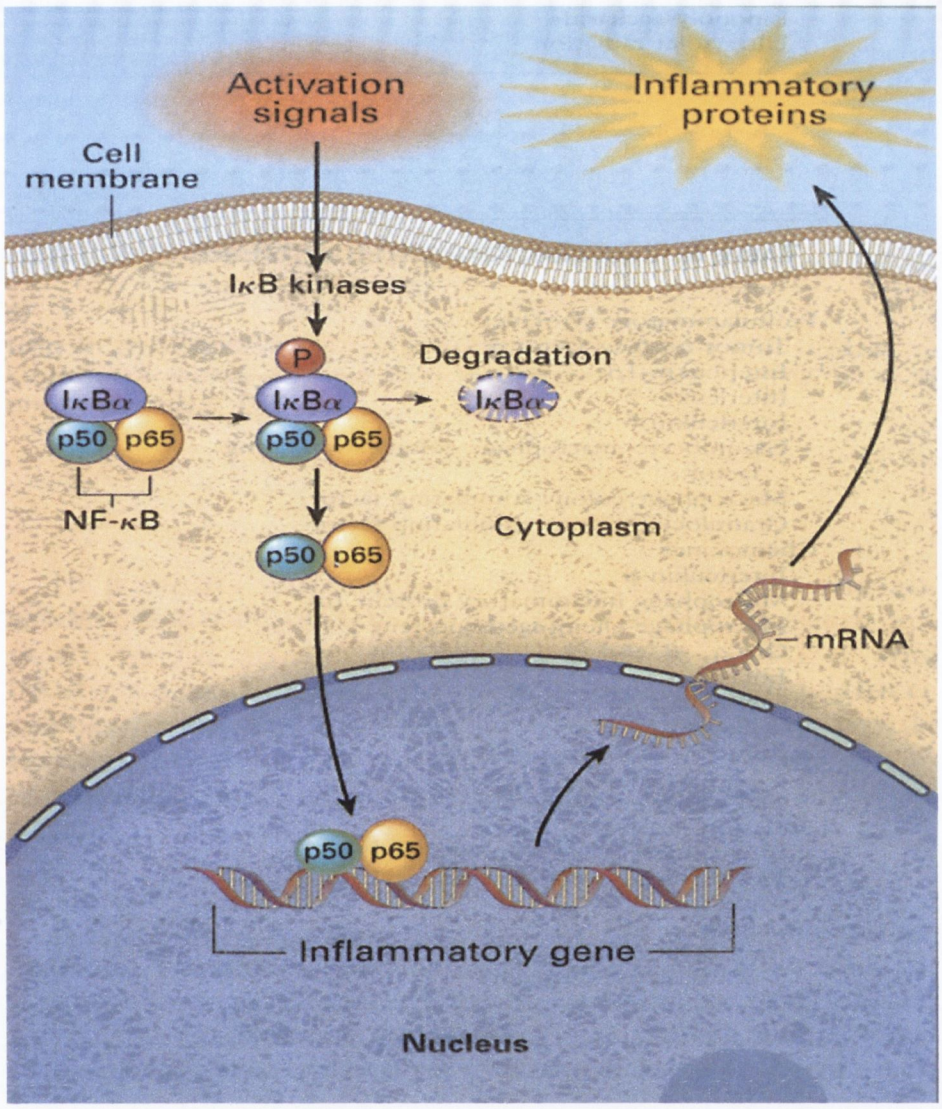


Figure 5.1 The cellular mechanism leading to the activation of NFκB

In unstimulated cells NFκB is localised within the cytoplasm by binding to IκBα and IκBβ and this prevents nuclear translocation. Activation of cells with LPS, cytokines, viruses, or oxidants results in the rapid proteosomal degradation of IκB with release of NFκB which in turn is translocated into the nucleus where it binds to specific sequences in the promoter regions of target genes responsible for the generation of proinflammatory cytokines, chemokines, enzymes that generate mediators of inflammation, immune receptors and adhesion molecules (Barnes *et al,* 1997).

Materials and methods

The human monocyte THP-1 cell line (European collection of cell cultures, Salisbury, UK) was cultured at 37°C in 5% CO_2 at a density of 2-9x10^5 per ml. Cell culture medium included was RPMI-1640 (Gibco BRL, UK) supplemented with 2mM glutamine (Sigma, UK), 10% fetal calf serum (FCS) (Gibco BRL, UK), penicillin 50units/ml and of streptomycin 50units/ml (Gibco BRL, UK). Cells were separated from medium every 36 hours and >90% viability confirmed by ethidium bromide acridine orange (Sigma, UK). Prior to experiment the cells were then resuspened at a concentration of 2x10^6/ml in culture medium which contained 0% and 10% FCS. The cells were then exposed to LPS 10μg/ml with or without preincubation with APC at different concentrations (200μg/ml, APC 100μg/ml, and APC 20μg/ml). The reaction was terminated after four hours by the addition of ice cold phosphate buffered saline (PBS) (Gibco BRL, UK). Following centrifugation at 1000g for 10 minutes, the supernatant was collected and stored at -70°C for subsequent analysis TNFα analysis. TNFα was measured in the supernatant fraction using the ELISA method (R&D Systems, UK) as previously described (chapter 3).

Preparation of nuclear extracts

Nuclear extracts were prepared from the cell pellet for measurement of NFκB (Mackman *et al*, 1991). The cells were lysed by exposure to a hypotonic solution and detergent (Nonindet, Sigma UK). The nuclei were then disrupted in a high salt buffer and the presence of PMSF (a protease inhibitor) and the experimental condition of 4°C inhibited nuclear protein degradation.

Buffers A, B and C were prepared as illustrated in table 5.1. The cell pellet was resuspended in 1ml of buffer A, transferred to eppendorf cup and placed on ice for 10 minutes. The samples were then microfuged at 300g for 10 minutes at 4°C. The supernatant was decanted and the pellet was resuspended in 20µl of buffer A containing 0.1% Nonidet (Sigma, UK) and incubated on ice 10 minutes. The samples were microfuged at 800g for 10 minutes at 4°C and the supernatant was decanted. The pellet was resuspended in 15µl of buffer B to lyse nucleus and incubated on ice for 15 min. The samples were microfuged at 800g for 10 minutes at 4°C and the supernatant which contained the nuclear extracts were removed and stored in 75µl of buffer C.

Buffer A

	Stock	Volume added	Final Concentration
HEPES pH 7.9	1M	100µl	10mM
$MgCl_2$	1M	15µl	1.5mM
KCL	1M	100µl	10mM
H_20		9785µl	

Immediately before experiment PMSF and DTT were added to buffer A to give a final concentration of 0.5mM PMSF and 0.5m M DTT.

Buffer B

	Stock	Volume added	Final Concentration
HEPES pH 7.9	1M	200µl	20mM
$MgCl_2$	1M	15µl	1.5mM
NaCl	5M	840µl	420mM
EDTA	0.5M	4µl	0.2mM
Glycerol	100%	2500µl	25%
H_20		6440µl	

Immediately before experiment PMSF was added to 200µl of buffer B resulting in a final concentration of 0.5mM PMSF.

Buffer C

	Stock	Volume added	Final Concentration
HEPES pH 7.9	1M	100µl	10mM
KCl	1M	500µl	50mM
NaCl	5M	840µl	420mM
EDTA	0.5M	4µl	0.2mM
Glycerol	100%	2000µl	20%
H_20		6556µl	

Immediately before experiment add 4µl of 1M PMSF and 4µl of DTT to 800µl of buffer C to give a final concentration of 0.5mM PMSF and 0.5mM DTT.

Table 5.1 Preparation of buffers used in nuclear extraction.

The determination of nuclear protein concentration

It is important that the same concentration of total nuclear protein is used in the measurement of nuclear NFκB from each experiment. This corrects for variation in cell number and nuclear protein extraction efficiency. The concentration of protein in the nuclear extracts was determined using the Bradford method (Bradford, 1976). Bradford reagents (Cromassie) are aromatic dyes that bind to proteins and cause a shift in the dye absorption maximum from 465 to 595 nm. The absorption at 595nm can be measured using a spectrophotometer and is directly proportional to the protein concentration. Standard solutions of protein were prepared by diluting bovine serum albumin (BSA) in H_2O resulting in final concentration of 0μg/ml, 2μg/ml, 4μg/ml, 6μg/ml, 8μg/ml and 10μg/ml of protein. 10μl of the nuclear extracts were diluted in 990μl of H_2O. 10μl of standards and samples were then mixed with 1ml of Bradford Reagent (Sigma). The concentration of protein was calculated from a standard curve by spectrophotometry at 595nm.

Electrophoretic Mobility Shift Assay (EMSA)

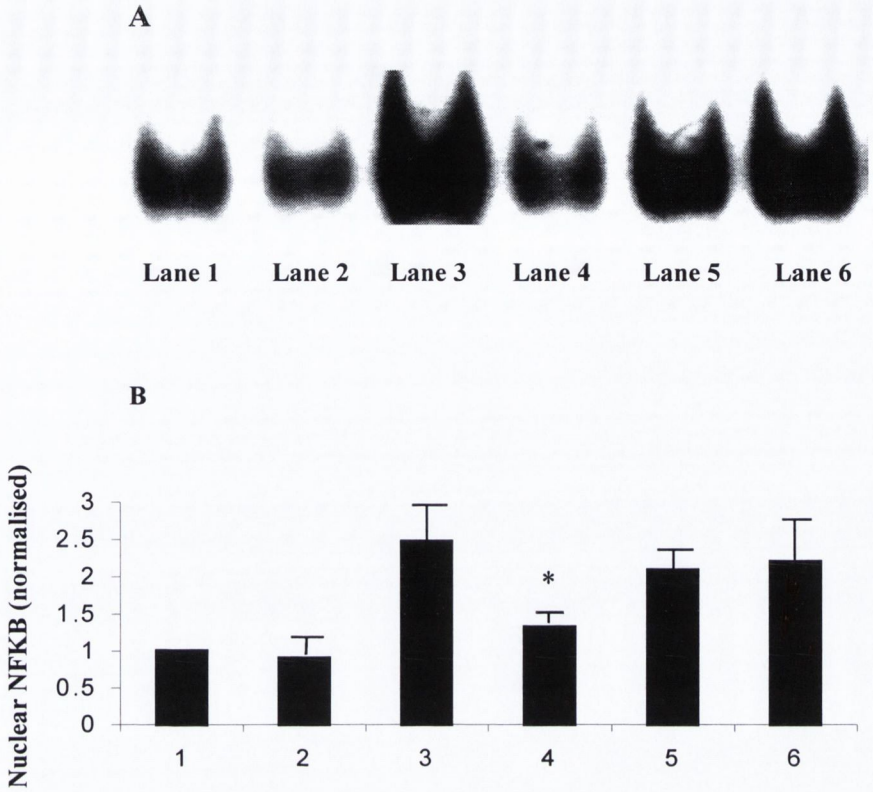
Binding of transcription factors to radiolabelled consensus sequence of DNA causes altered electrophoretic mobility in a polyacrylamide gel. DNA bound to transcription factors can be separated from unbound DNA by electrophoresis and visualised by exposure onto an autoradiographic film. The presence of excess DNA competitor (poly(dI-dC)·poly(dI-dC)) prevents non-specific binding of transcription factors to the DNA probe.

An oligonucleotide (Promega, UK) containing the κB binding site (underlined) 5'-AGTTGAGGGGACTTTCCCAGGC-3' was annealed to a complementary primer and endlabelled using the T4 polynucleotide kinase (Promega, UK) which exchanges a phosphate group at the 5' end of the double stranded DNA with radiolabelled phosphate group [γ^{32}P]dATP (Amersham). Binding reactions were performed in a 20μl volume containing 4μg of nuclear extract, 2μg of poly(dIdC)·poly(dI-dC)) (Amersham Pharmacia, UK), 1 μl of labelled oligonucleotide (10,000 cpm), 2μl of binding buffer (100mM Tris pH 7.5, 1M NaCl, 50mM DTT, 10mM EDTA, 40% glycerol (BDH, UK)) and 1 mg/ml nuclease free BSA. After a 30-minute binding reaction, at room temperature, the protein-DNA complexes were separated from the free DNA probe by electrophoresis and visualised on a 4% nondenaturing acrylamide gel run on 0.5 X Tris-borate-EDTA buffer (89mM Tris-Hcl, 89mM Boric acid, 2mM EDTA). Gels were dried and visualised by autoradiography. Band intensities were quantified by densitometric analysis using a Pharmacia LKB Imagemaster DTS densitometer and Diversity one™ software.

Statistical analysis: All experiments were performed in triplicate. The percentage inhibition of NFκB and TNFα was evaluated for statistical analysis by the Mann Whitney U test.

Results

Treatment of THP-1 cells with 100ng/ml of LPS strongly activated NFκB in THP-1 cells (figure 5.2, compare lanes 3 and 1). Pre-incubation with 200μg of APC completely blocked the effect of LPS (compare lanes 4 and 3) and partially reduced basal NFκB (compare lanes 2 and 1). A more marginal effect was evident at 100μg/ml of APC (lane 5) and no effect was seen with 20μg/ml of APC. Inhibition of NFκB was demonstrated both in the presence and absence of FCS (not shown). The inhibition of NFκB was associated with a significant reduction in the concentration of TNFα in the supernatant (figure 3.1). However, APC did not inhibit the activation of NFκB associated with the higher dose of LPS 10μg/ml (figure 5.3). The failure of APC to inhibit NFκB at the higher dose of LPS occurred despite significant inhibition of supernatant TNFα in the same experiment (figure 3.1).

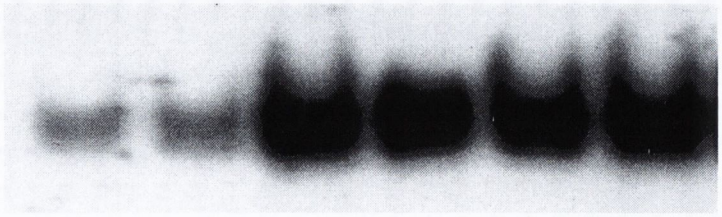


Lane 1 = Resting, Lane 2 = APC 200μg/ml, Lane 3 = LPS 100ng/ml, Lane 4 = LPS 100ng/ml + APC 200μg/ml, Lane 5 = LPS 100ng/ml + APC 100μg/ml, Lane 6 = LPS 100ng/ml + APC 20μg/ml

Figure 5.2 APC inhibits nuclear translocation of NFκB after stimulation with LPS 100ng/ml.

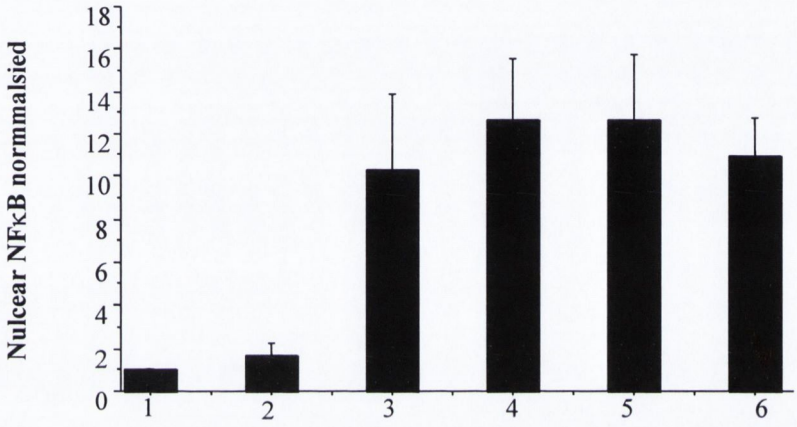
Nuclear extracts were isolated from THP-1 cells pre-treated APC 200μg/ml, 100μg/ml and 20μg/ml for 10 minutes prior to the addition of LPS 100ng/ml for four hours. The nuclear concentration of NFκB was assessed by electrophoretic mobility shift assay (EMSA) (A). Band intensities were then quantified by densitometric analysis. Band intensities were normalised against values obtained with resting cells. Data is expressed as the mean +/-1 SD for of triplicate experiments (B). *p <0.05, determined by Mann Whitney U test.

A



Lane 1 Lane 2 Lane 3 Lane 4 Lane 5 Lane 6

B



Lane 1 = Resting, Lane 2 = APC 200μg/ml, Lane 3 = LPS 10μg/ml, Lane 4 =LPS 10μg/ml + APC 200μg/ml, Lane 5 = LPS 10μg/ml + APC 100μg/ml, Lane 6 = LPS 10μg/ml + APC 20μg/ml

Figure 5.3 APC does not inhibit nuclear translocation of NFκB after stimulation with LPS 10μg/ml. Nuclear extracts were isolated from THP-1 cells pre-treated APC 200μg/ml, 100μg/ml and 20μg/ml for 10 minutes prior to the addition of LPS 10μg/ml for four hours. The concentration of NFκB was assessed by EMSA (A). APC had no effect on LPS induced NFκB despite significant inhibition of supernatant TNFα. Band intensities were then quantified by densitometric analysis and the results were normalised against values obtained with resting cells. Data is expressed as the mean +/- 1 SD of triplicate experiments (B).

Discussion

In this study we have demonstrated that APC inhibits LPS induced activation of NFκB at the lower concentration of LPS (100ng/ml). However, APC did not inhibit NFκB at the higher concentration of LPS (10μg/ml) despite a significant reduction in TNFα mRNA and supernatant TNFα. Therefore, it appears that the ability of APC to inhibit TNFα mRNA is not solely dependant on the inhibition of the nuclear translocation of NFκB. Additional mechanism(s) must also be involved and these are likely to include inhibition of nuclear translocation of Egr-1 and c-Jun, or reduced mRNA stability.

The inhibition of NFκB, even though it only occurs at the lower concentration of LPS, is highly significant given the importance of this transcription factor in the inflammatory response. The ability of APC to inhibit NFκB may globally downregulate the production of a wide variety of pro-inflammatory molecules, which are important at various stages in the evolution of the inflammatory response in sepsis. It is this particular property of NFκB that makes it such an attractive molecular target for novel anti-inflammatory therapies and offers an explanation for protective effect of APC in animal models of sepsis and the apparent reduction in mortality associated with PC replacement therapy in patients with severe meningococcaemia. The demonstration of an apparent threshold effect of LPS, above which APC is unable to inhibit NFκB *in vitro,* suggests that APC may lose an important component of it's anti-inflammatory effect above a certain level of circulating endotoxin.

The activation of NFκB has also been implicated in a wide range of other disease states including malignancy and chronic inflammatory disorders such as rheumatoid arthritis, asthma and ulcerative colitis (Barnes & Karin, 1997; Bohrer *et al*, 1997; Bohrer *et al*, 1997). Furthermore NFκB is a critical transcription factor in the generation of tissue factor within monocytes and endothelial cells (Hall *et al*, 1999). Tissue factor expressed at the site of vessel injury binds to factor VII resulting the generation of the TF: Factor VIIa complex. This complex is the primary initiator of the coagulation cascade, *in vivo,* and plays a pivotal role in the development myocardial infarction and ischaemic stroke (Tremoli *et al*, 1999). Therefore, the ability of APC to inhibit the activation of NFκB, identifies a molecular target for potential anti-thrombotic as well as anti-inflammatory therapies. In addition, inherited or acquired defects within this pathway may predispose to the development of a variety of disease states. Further work is required to elucidate the mechanism by which APC mediates the inhibition of NFκB and to determine the cellular pathway(s), which are responsible for the inhibition of TNFα at the higher concentration of LPS.

Chapter 6

Discussion

The clinical relevance of the dual functional properties of PC is illustrated by the development of severe APCD deficiency in meningococcaemia which is associated with the development of purpura fulminans and cytokine driven multiorgan failure. Protein C replacement therapy was associated with a reduction in the predicted morbidity and mortality. We accept that many physicians may require a randomised prospective placebo controlled trial prior to the widespread use of PC replacement therapy in meningococcal disease. However, such a trial may prove impossible to perform. We know that the significant morbidity encountered in severe meningococcaemia is secondary to widespread microvascular thrombosis resulting in tissue necrosis, which in the majority of patients leads to significant complications including skin grafting and amputations. It is also increasingly apparent that failure of the PC pathway is the primary cause of purpura fulminans (Smith & White, 1999). While a combination of interactive factors are likely to contribute to the development of purpura fulminans in meningococcaemia, it is likely that the severe reduction in PC represents the dominant lesion. Protein C deficiency is the only proven defect within the PC pathway, the level is predictive of outcome and PC replacement is associated with a reduction in predicted morbidity. Furthermore, the administration of PC or APC are the only therapeutic strategies, that are currently available, to correct PC pathway failure, which is central to the development of purpura fulminans. APC has the advantage of not requiring endogenous activation, however, it is more likely to be associated with significant haemorrhagic complications and therefore, until proven superior to PC, should be used with caution. These observations along with the data

presented in this thesis support the use of early PC replacement therapy in patients with severe disease (Smith *et al*, 1997; Kreuz *et al*, 1998; Rintala *et al*, 1998; Rivard *et al*, 1995). When one considers the imminent morbidity associated with the rapid development of widespread thrombotic lesions in severe purpura fulminans, it might prove difficult to randomise such patients when the outcome of the placebo arm of a such a study is already known and includes, skin grafting, amputation and possibly death (Havens *et al*, 1989; Giraud *et al*, 1991; Fijnvandraat *et al*, 1995; Powars *et al*, 1993; Ramsay *et al*, 1997; Genoff *et al*, 1992).

Prior to using PC replacement therapy a number of clinically relevant points should be considered. As with any novel adjunctive therapeutic strategy, the addition of PC replacement therapy is unlikely to be of benefit if the standard management of patients is suboptimal. Failure to adhere to the basic principles of management of meningococcaemia will compromise the patient outcome and invalidate any potential benefits from PC replacement. Established therapeutic interventions include early administration of antibiotics and aggressive volume expansion with repeated boluses of intravenous colloids (40 ml/kg) given prior to ionotropic drugs to secure circulation to vital organs (Astic 1998). Correction of acid-base and electrolyte abnormalities is also essential (Astic 1998). It appears that early use of oxygen and mechanical ventilation is of benefit even when gas exchange is adequate (Asric 1998). While PC is effective at reversing the coagulopathy, it does not obviate the need for standard coagulation support. We accept that the trigger for correction of fibrinogen in DIC is usually 0.8-1.0g/l, however, we believe a more aggressive approach regarding fibrinogen

replacement is required in severe meningococcaemia. Fibrinogen consumption may be rapid in the first twenty-four hours, and patients with purpura fulminans frequently receive heparin either for CVVHF or in an attempt to limit microvascular thrombosis. We aim to maintain the platelet count greater than $50x10^9$/l and fibrinogen greater than 2.0g/l, in all patients, prior to and during the first twenty-four hours of PC replacement therapy.

The decision to treat with PC concentrate should be based on; the level of circulating PC, the severity of the associated coagulopathy and the clinical picture i.e. purpuric lesions that are rapidly developing, impending peripheral gangrene and deterioration in vital organ function. It is clear from the data presented in this thesis that patients will continue to die and require amputations despite early replacement therapy. It is likely that PC replacement will have little impact upon the mortality or morbidity associated with meningoencephalopathy and may also fail to save those patients who are premorbid at the time of admission to hospital. Furthermore early PC replacement may, in some cases, fail to prevent the complications associated with purpura fulminans. This may reflect the inability of the PC pathway to lyse established thrombi or the presence of additional prothrombotic defects. It is possible that this subgroup of patients would benefit from the administration of APC, however, it is unclear how these treatment failures could be identified in advance.

The elucidation of the molecular mechanism responsible for the anti-inflammatory properties of APC has assumed greater clinical significance with the apparent reduction

in mortality associated with PC replacement in severe meningococcaemia. The ability of APC to inhibit endotoxin induced TNFα production is likely to be highly significant given the important role of the later in the host inflammatory response in sepsis. However, there is no published data on the cellular mechanisms responsible for this effect. It has been suggested from binding studies that a receptor for PC exists on monocytes, although this receptor has yet to be characterised. We have demonstrated that APC inhibits TNFα mRNA and the critical pro-inflammatory transcription factor NFκB. These experiments were performed on a THP-1 monocytic cell line and will need to be repeated using monocytes collected from whole blood. However, THP-1 cells have been previously validated as an accurate model of the monocyte response to endotoxin and it is likely that similar effects will be demonstrated in peripheral blood monocytes (Yao *et al* , 1997). The ability of PC to exhibit similar anti-inflammatory properties to APC contradicts previously published data (Grey *et al*, 1994). It is difficult to explain these discrepant results, although, it may reflect the differences in the purification process or the viral inactivation step used in the preparation of the PC concentrate. This discrepancy clearly requires further study, as it may influence the efficacy of different PC concentrates in sepsis. We used the same PC concentrate for the *in vitro* experiments as was administered to the patients with severe meningococcaemia.

A considerable body of evidence has been uncovered linking inflammation and coagulation. An inflammatory lesion within the atherosclerotic plaque is responsible for its subsequent rupture and the development of coronary artery thrombosis (Ross, 1999).

C reactive protein, interleukin 6, ICAM-1 and the acute phase coagulation proteins PAI-1, fibrinogen, and FVIII have all been associated with an increased risk of arterial thrombosis (Huber *et al* 1999; Ridker *et al* 1998; Ridker *et al* 1997; Meade *et al* 1986). Furthermore, recent data has demonstrated that thrombin, tissue factor, factor VIIa, and factor Xa can all activate cells directly, mediated in part by the cleavage of cell surface protease activated receptors (PAR's), resulting in a range of pro-inflammatory effects (Johnson *et al*, 1998; Kahn *et al*, 1999; Jones & Geczy, 1990; Cunningham *et al*, 1999; Camerer *et al*, 1999; Camerer *et al*, 1996; Cirino *et al*, 1997). In addition, AT appears to have potent anti-inflammatory properties and is protective in animal models of sepsis (Liu *et al*, 1998; Kaplanski *et al*, 1997; Hoffman & Cooper, 1995; Naldini *et al*, 1993). The demonstration that APC and PC replacement are protective in animal models of endotoxic shock and in severe meningococcaemia respectively (Taylor *et al*, 1987; Smith *et al*, 1997), coupled with the ability of APC/PC to inhibit TNFα (mRNA and protein) and NFκB, is further compelling evidence linking the cascades of inflammation and coagulation. It is possible that these pathways may have evolved from a common ancestral origin and that the development of severe acquired PC deficiency may represent a primitive response to infection. This would serve to localise the infective organisms within microvascular thromboses and at the same time boost the inflammatory response by reducing a negative regulator of pro-inflammatory cytokine production. This hypothesis is supported by the high concentration of meningococal organisms within the purpuric skin lesions, a finding which has been utilised in the development of a diagnostic assay for meningococcaemia (van Deuren *et al*, 1993). Furthermore, while excessive levels of TNFα may be harmful, it is also clear

that this pro-inflammatory cytokine is an important component of host defence and enhances survival by stimulating effector cell microbicidal activity (Cross *et al*, 1989; Cross *et al*, 1995). In certain circumstances it may be advantageous to boost a sub-optimal TNFα response to infection. Therefore, the development of acquired PC deficiency in severe meningococcaemia may represent a response that initially evolved as a protective mechanism.

In conclusion, the APC/PC appears to have both anti-inflammatory and anticoagulant properties. The ability of APC/PC to inhibit such a pleotropic transcription factor as NFκB suggests that inhibition of TNFα may represent only one component of its anti-inflammatory properties. It is likely that the pioneering work, which initially identified the protective effects of APC in animal models of sepsis, will eventually lead to a completely new understanding of the role of the PC pathway in the regulation of inflammation and in the pathophysiology of a wide variety of immune mediated diseases. Furthermore, the elucidation of the anti-inflammatory pathways by which the PC pathway regulates inflammation will provide the opportunity to identify at risk individuals and develop novel therapeutic strategies to modulate aberrant immune responses.

Inflammation in itself is not to be considered as a disease……, where it can alter the disease modes of action, it leads to cure; but where it cannot accomplish that solitary purpose..... it does mischief'.

John Hunter: Treatise on the blood, inflammation and gun shot wounds, London, 1794.

Future studies

1. **Determine the mechanism(s) by which APC inhibits TNFα mRNA and NFκB.**

Further studies are required to confirm that PC/APC inhibits TNFα mRNA and NFκB in peripheral blood monocytes and to compare the anti-inflammatory effects of different PC preparations. Further work is also required to identify the exact site of action of APC by assessing its effect on the MAPKinase pathways, Egr-1 and c-Jun transcription factors, TNFα mRNA stability and the cytoplasmic release of TNF α.

2. **Determine the effects of APC on tissue factor expression and on the generation of a wide variety of NFκB mediated pro-inflammatory molecules.**

The ability of APC to inhibit NFκB raises the possibility that it may also downregulate tissue factor expression and a variety of pro-inflammatory molecules. The inhibition of tissue factor would be highly significant given the pivotal role that it plays in the development of myocardial infarction and ischaemic stroke. Furthermore the ability of APC to downregulate a range of pro-inflammatory molecules would greatly enhance it's importance in the regulation of the inflammatory response.

3. **The development of more effective strategies for severe meningococcal infection.**

Additional therapeutic strategies are required for those patients who fail to respond to PC replacement therapy. Treatment options include the co-administration of recombinant soluble thrombomodulin to provide endogenous PC activation or the use of APC infusions. The future management of patients with severe meningococcal infection

will probably include a cocktail of therapies selected on the basis of individual patient requirements and designed to correct the pro-inflammatory and procoagulant complications at various stages in their evolution. Since the coagulation system plays a critical role in the pathophysiology of the complications of severe meningococcaemia, it is imperative that coagulation specialists take an active role in the management of these patients and in the development and assessment of novel therapeutic strategies.

Reference List

Abraham, E., Wunderink, R., Silverman, H., Perl, T.M., Nasraway, S., Levy, H., Bone, R., Wenzel, R.P., Balk, R. & Allred, R. (1995) Efficacy and safety of monoclonal antibody to human tumor necrosis factor alpha in patients with sepsis syndrome. A randomized, controlled, double-blind, multicenter clinical trial. TNF-alpha MAb Sepsis Study Group. *JAMA*, **273**, 934-941.

Allaart, C.F., Poort, S.R., Rosendaal, F.R., Reitsma, P.H., Bertina, R.M. & Briet, E. (1993) Increased risk of venous thrombosis in carriers of hereditary protein C deficiency defect [see comments]. *Lancet*, **341**, 134-138.

Astic ME. (1998) Septic shock. *Lancet* **352,** 1501-1505..

Attanasio, M., Gori, A.M., Giusti, B., Pepe, G., Comeglio, P., Brunelli, T., Prisco, D., Abbate, R., Gensini, G.F. & Neri, S.G. (1998) Cytokine gene expression in human LPS- and IFNgamma-stimulated mononuclear cells is inhibited by heparin. *Thromb Haemost*, **79**, 959-962.

Baldwin, A.S.J. (1996) The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol*, **14**, 649-683.

Barnes, P.J. & Karin, M. (1997) Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med*, **336**, 1066-1071.

Barnes, P.J. & Karin, M. (1997) Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med*, **336**, 1066-1071.

Beutler, B., Milsark, I.W. & Cerami, A.C. (1985) Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. *Science*, **229**, 869-871.

Bodmer, M., Fournel, M.A. & Hinshaw, L.B. (1993) Preclinical review of anti-tumor necrosis factor monoclonal antibodies. *Crit Care Med*, **21**, S441-S446

Bohrer, H., Qiu, F., Zimmermann, T., Zhang, Y., Jllmer, T., Mannel, D., Bottiger, B.W., Stern, D.M., Waldherr, R., Saeger, H.D., Ziegler, R., Bierhaus, A., Martin, E. & Nawroth, P.P. (1997) Role of NFkappaB in the mortality of sepsis. *J Clin Invest*, **100**, 972-985.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, **72**, 248-254.

Branson, H.E., Katz, J., Marble, R. & Griffin, J.H. (1983) Inherited protein C deficiency and coumarin-responsive chronic relapsing purpura fulminans in a newborn infant. *Lancet*, **2**, 1165-1168.

Camerer, E., Rottingen, J.A., Gjernes, E., Larsen, K., Skartlien, A.H., Iversen, J.G. & Prydz, H. (1999) Coagulation factors VIIa and Xa induce cell signaling leading to up- regulation of the egr-1 gene. *J Biol Chem*, **274**, 32225-32233.

Camerer, E., Rottingen, J.A., Iversen, J.G. & Prydz, H. (1996) Coagulation factors VII and X induce Ca2+ oscillations in Madin-Darby canine kidney cells only when proteolytically active. *J Biol Chem*, **271**, 29034-29042.

Chollet-Martin, S., Montravers, P., Gibert, C., Elbim, C., Desmonts, J.M., Fagon, J.Y. & Gougerot-Pocidalo, M.A. (1992) Subpopulation of hyperresponsive polymorphonuclear neutrophils in patients with adult respiratory distress syndrome. Role of cytokine production. *Am Rev Respir Dis* , **146**, 990-996.

Cirino, G., Cicala, C., Bucci, M., Sorrentino, L., Ambrosini, G., DeDominicis, G. & Altieri, D.C. (1997) Factor Xa as an interface between coagulation and inflammation. Molecular mimicry of factor Xa association with effector cell protease receptor-1 induces acute inflammation in vivo [see comments]. *J Clin Invest*, **99**, 2446-2451.

Conard, J., Horellou, M.H., Teger-Nilsson, A.C., Bertina, R.M. & Samama, M. (1984) The fibrinolytic system in patients with congenital protein C deficiency. *Thromb Res*, **36**, 363-367.

Connolly, A.R., Cleland, L.G. & Kirkham, B.W. (1995) Mathematical considerations of competitive polymerase chain reaction. *J Immunol Methods*, **187**, 201-211.

Cowley, H.C., Heney, D., Gearing, A.J., Hemingway, I. & Webster, N.R. (1994) Increased circulating adhesion molecule concentrations in patients with the systemic inflammatory response syndrome: a prospective cohort study. *Crit Care Med*, **22**, 651-657.

Cross, A., Asher, L., Seguin, M., Yuan, L., Kelly, N., Hammack, C., Sadoff, J. & Gemski, P.J. (1995) The importance of a lipopolysaccharide-initiated, cytokine-mediated host defense mechanism in mice against extraintestinally invasive Escherichia coli. *J Clin Invest*, **96**, 676-686.

Cross, A.S., Sadoff, J.C., Kelly, N., Bernton, E. & Gemski, P. (1989) Pretreatment with recombinant murine tumor necrosis factor alpha/cachectin and murine interleukin 1 alpha protects mice from lethal bacterial infection. *J Exp Med*, **169**, 2021-2027.

Cunningham, M.A., Romas, P., Hutchinson, P., Holdsworth, S.R. & Tipping, P.G. (1999) Tissue factor and factor VIIa receptor/ligand interactions induce proinflammatory effects in macrophages. *Blood*, **94**, 3413-3420.

Derkx, H.H., Kuijper, E.J., Fijen, C.A., Jak, M., Dankert, J. & van Deventer, S.J. (1995) Inherited complement deficiency in children surviving fulminant meningococcal septic shock [see comments]. *Eur J Pediatr*, **154**, 735-738.

DiDonato, J., Mercurio, F., Rosette, C., Wu-Li, J., Suyang, H., Ghosh, S. & Karin, M. (1996) Mapping of the inducible IkappaB phosphorylation sites that signal its ubiquitination and degradation. *Mol Cell Biol*, **16**, 1295-1304.

Drake, T.A., Cheng, J., Chang, A. & Taylor, F.B.J. (1993) Expression of tissue factor, thrombomodulin, and E-selectin in baboons with lethal Escherichia coli sepsis [published erratum appears in Am J Pathol 1993 Aug;143(2):649]. *Am J Pathol*, **142**, 1458-1470.

Dreyfus, M., Magny, J.F., Bridey, F., Schwarz, H.P., Planche, C., Dehan, M. & Tchernia, G. (1991) Treatment of homozygous protein C deficiency and neonatal purpura fulminans with a purified protein C concentrate. *N Engl J Med*, **325**, 1565-1568.

Endo, S., Inada, K., Kasai, T., Takakuwa, T., Yamada, Y., Koike, S., Wakabayashi, G., Niimi, M., Taniguchi, S. & Yoshida, M. (1995) Levels of soluble adhesion molecules and cytokines in patients with septic multiple organ failure. *J Inflamm*, **46**, 212-219.

Esmon, C.T. (1989) The roles of protein C and thrombomodulin in the regulation of blood coagulation. *J Biol Chem*, **264**, 4743-4746.

Esmon, C.T. (1994) Possible involvement of cytokines in diffuse intravascular coagulation and thrombosis. *Baillieres Clin Haematol*, **7**, 453-468.

Esmon, C. T. (1999) Endothelial protein C receptor. *Thromb Haemost* **82(2),** 251-258.

Esmon, C.T., Taylor, F.B.J. & Snow, T.R. (1991) Inflammation and coagulation: linked processes potentially regulated through a common pathway mediated by protein C. *Thromb Haemost*, **66**, 160-165.

Fenton, M.J. & Golenbock, D.T. (1998) LPS-binding proteins and receptors. *J Leukoc Biol*, **64**, 25-32.

Fijnvandraat, K., Derkx, B., Peters, M., Bijlmer, R., Sturk, A., Prins, M.H., van Deventer, S.J. & ten Cate, J.W. (1995) Coagulation activation and tissue necrosis in meningococcal septic shock: severely reduced protein C levels predict a high mortality. *Thromb Haemost*, **73**, 15-20.

Fijnvandraat, K., Peters, M., Derkx, B., van Deventer, S. & ten Cate, J.W. (1994) Endotoxin induced coagulation activation and protein C reduction in meningococcal septic shock. *Prog Clin Biol Res*, **388**, 247-254.

Fourrier, F., Chopin, C., Goudemand, J., Hendrycx, S., Caron, C., Rime, A., Marey, A. & Lestavel, P. (1992) Septic shock, multiple organ failure, and disseminated intravascular coagulation. Compared patterns of antithrombin III, protein C, and protein S deficiencies [see comments]. *Chest*, **101**, 816-823.

Fukudome, K. & Esmon, C.T. (1994) Identification, cloning, and regulation of a novel endothelial cell protein C/activated protein C receptor. *J Biol Chem*, **269**, 26486-26491.

Gaffney, P.R., Doyle, C.T., Gaffney, A., Hogan, J., Hayes, D.P. & Annis, P. (1995) Paradoxical response to heparin in 10 patients with ulcerative colitis. *Am J Gastroenterol*, **90** , 220-223.

Gamm, H., Lindemann, A., Mertelsmann, R. & Herrmann, F. (1991) Phase I trial of recombinant human tumour necrosis factor alpha in patients with advanced malignancy. *Eur J Cancer*, **27**, 856-863.

Genoff, M.C., Hoffer, M.M., Achauer, B. & Formosa, P. (1992) Extremity amputations in meningococcemia-induced purpura fulminans. *Plast Reconstr Surg*, **89**, 878-881.

Giraud, T., Dhainaut, J.F., Schremmer, B., Regnier, B., Desjars, P., Loirat, P., Journois, D. & Lanore, J.J. (1991) Adult overwhelming meningococcal purpura. A study of 35 cases, 1977- 1989. *Arch Intern Med*, **151**, 310-316.

Gironell, A., Domingo, P., Mancebo, J., Coll, P. & Marti-Vilalta, J.L. (1995) Hemorrhagic stroke as a complication of bacterial meningitis in adults: report of three cases and review. *Clin Infect Dis*, **21**, 1488-1491.

Grey, S., Hau, H., Salem, H.H. & Hancock, W.W. (1993) Selective effects of protein C on activation of human monocytes by lipopolysaccharide, interferon-gamma, or PMA: modulation of effects on CD11b and CD14 but not CD25 or CD54 induction. *Transplant Proc*, **25**, 2913-2914.

Grey, S.T., Tsuchida, A., Hau, H., Orthner, C.L., Salem, H.H. & Hancock, W.W. (1994) Selective inhibitory effects of the anticoagulant activated protein C on the responses of human mononuclear phagocytes to LPS, IFN-gamma, or phorbol ester. *J Immunol*, **153**, 3664-3672.

Hall, A.J., Vos, H.L. & Bertina, R.M. (1999) Lipopolysaccharide induction of tissue factor in THP-1 cells involves jun protein phosphorylation and nuclear factor kappaB nuclear translocation [In Process Citation]. *J Biol Chem*, **274**, 376-383.

Halstensen, A., Ceska, M., Brandtzaeg, P., Redl, H., Naess, A. & Waage, A. (1993) Interleukin-8 in serum and cerebrospinal fluid from patients with meningococcal disease. *J Infect Dis*, **167**, 471-475.

Han, J., Brown, T. & Beutler, B. (1990) Endotoxin-responsive sequences control cachectin/tumor necrosis factor biosynthesis at the translational level [published erratum appears in J Exp Med 1990 Mar 1;171(3):971-2]. *J Exp Med*, **171**, 465-475.

Hancock, W.W., Grey, S.T., Hau, L., Akalin, E., Orthner, C., Sayegh, M.H. & Salem, H.H. (1995) Binding of activated protein C to a specific receptor on human

mononuclear phagocytes inhibits intracellular calcium signaling and monocyte-dependent proliferative responses. *Transplantation*, **60**, 1525-1532.

Havens, P.L., Garland, J.S., Brook, M.M., Dewitz, B.A., Stremski, E.S. & Troshynski, T.J. (1989) Trends in mortality in children hospitalized with meningococcal infections, 1957 to 1987. *Pediatr Infect Dis J*, **8**, 8-11.

Heeb, M.J., Gruber, A. & Griffin, J.H. (1991) Identification of divalent metal ion-dependent inhibition of activated protein C by alpha 2-macroglobulin and alpha 2-antiplasmin in blood and comparisons to inhibition of factor Xa, thrombin, and plasmin. *J Biol Chem*, **266**, 17606-17612.

Hermans, P.W., Hibberd, M.L., Booy, R., Daramola, O., Hazelzet, J.A., de Groot, R. & Levin, M. (1999) 4G/5G promoter polymorphism in the plasminogen-activator-inhibitor-1 gene and outcome of meningococcal disease. Meningococcal Research Group. *Lancet*, **354**, 556-560.

Heyderman, R.S. (1993) Sepsis and intravascular thrombosis. *Arch Dis Child*, **68**, 621-623.

Hoffman, M. & Cooper, S.T. (1995) Thrombin enhances monocyte secretion of tumor necrosis factor and interleukin-1 beta by two distinct mechanisms. *Blood Cells Mol Dis*, **21**, 156-167.

Huber, S.A., Sakkinen, P., Conze, D., Hardin, N. & Tracy, R. (1999) Interleukin-6 exacerbates early atherosclerosis in mice. *Arterioscler Thromb Vasc Biol*, **19**, 2364-2367.

Huskisson, E.C. & Hart, F.D. (1969) Fulminating meningococcal septicaemia presenting with subarachnoid haemorrhage. *Br Med J*, **2**, 231-232.

Johnson, K., Choi, Y., DeGroot, E., Samuels, I., Creasey, A. & Aarden, L. (1998) Potential mechanisms for a proinflammatory vascular cytokine response to coagulation activation. *J Immunol*, **160**, 5130-5135.

Jones, A. & Geczy, C.L. (1990) Thrombin and factor Xa enhance the production of interleukin-1. *Immunology*, **71**, 236-241.

Jones, A.L. & Selby, P. (1989) Tumour necrosis factor: clinical relevance. *Cancer Surv*, **8**, 817-836.

Kahn, M.L., Nakanishi-Matsui, M., Shapiro, M.J., Ishihara, H. & Coughlin, S.R. (1999) Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *J Clin Invest*, **103**, 879-887.

Kaplanski, G., Fabrigoule, M., Boulay, V., Dinarello, C.A., Bongrand, P., Kaplanski, S. & Farnarier, C. (1997) Thrombin induces endothelial type II activation in vitro:

IL-1 and TNF- alpha-independent IL-8 secretion and E-selectin expression. *J Immunol*, **158** , 5435-5441.

Kirsch, E.A., Barton, R.P., Kitchen, L. & Giroir, B.P. (1996) Pathophysiology, treatment and outcome of meningococcemia: a review and recent experience [see comments]. *Pediatr Infect Dis J*, **15**, 967-978.

Kopp, E.B. & Ghosh, S. (1995) NF-kappa B and rel proteins in innate immunity. *Adv Immunol*, **58**, 1-27.

Kornelisse, R.F., Hazelzet, J.A., Savelkoul, H.F., Hop, W.C., Suur, M.H., Borsboom, A.N., Risseeuw-Appel, I.M., Van, d., V & de Groot, R. (1996) The relationship between plasminogen activator inhibitor-1 and proinflammatory and counterinflammatory mediators in children with meningococcal septic shock. *J Infect Dis*, **173**, 1148-1156.

Kovacich, J.C., Boyle, E.M.J., Morgan, E.N., Canty, T.G.J., Farr, A.L., Caps, M.T., Frank, N., Pohlman, T.H. & Verrier, E.D. (1999) Inhibition of the transcriptional activator protein nuclear factor kappaB prevents hemodynamic instability associated with the whole-body inflammatory response syndrome. *J Thorac Cardiovasc Surg*, **118**, 154-162.

Kreuz, W., Veldman, A., Escuriola-Ettingshausen, C., Schneider, W. & Beeg, T. (1998) Protein-C concentrate for meningococcal purpura fulminans [letter; comment]. *Lancet*, **351**, 986-987.

Kuppermann, N., Inkelis, S.H. & Saladino, R. (1994) The role of heparin in the prevention of extremity and digit necrosis in meningococcal purpura fulminans [see comments]. *Pediatr Infect Dis J*, **13**, 867-873.

Kurosawa, S., Stearns-Kurosawa, D.J., Carson, C.W., D'Angelo, A., Della, V.P. & Esmon, C.T. (1998) Plasma levels of endothelial cell protein C receptor are elevated in patients with sepsis and systemic lupus erythematosus: lack of correlation with thrombomodulin suggests involvement of different pathological processes [letter]. *Blood*, **91**, 725-727.

Liu, S., Khemlani, L.S., Shapiro, R.A., Johnson, M.L., Liu, K., Geller, D.A., Watkins, S.C., Goyert, S.M. & Billiar, T.R. (1998) Expression of CD14 by hepatocytes: upregulation by cytokines during endotoxemia. *Infect Immun*, **66**, 5089-5098.

Liu, S.F., Ye, X. & Malik, A.B. (1997) In vivo inhibition of nuclear factor-kappa B activation prevents inducible nitric oxide synthase expression and systemic hypotension in a rat model of septic shock. *J Immunol*, **159**, 3976-3983.

Mackman, N., Brand, K. & Edgington, T.S. (1991) Lipopolysaccharide-mediated transcriptional activation of the human tissue factor gene in THP-1 monocytic cells requires both activator protein 1 and nuclear factor kappa B binding sites. *J Exp Med*, **174**, 1517-1526.

Marlar, R.A., Endres-Brooks, J. & Miller, C. (1985) Serial studies of protein C and its plasma inhibitor in patients with disseminated intravascular coagulation. *Blood*, **66**, 59-63.

McGehee, W.G., Rapaport, S.I. & Hjort, P.F. (1967) Intravascular coagulation in fulminant meningococcemia. *Ann Intern Med*, **67**, 250-260.

Meade, T.W., Mellows, S., Brozovic, M., Miller, G.J., Chakrabarti, R.R., North, W.R., Haines, A.P., Stirling, Y., Imeson, J.D. & Thompson, S.G. (1986) Haemostatic function and ischaemic heart disease: principal results of the Northwick Park Heart Study [see comments]. *Lancet*, **2**, 533-537.

Michieletto, P. & Summonti, D. (1971) [On a case of subarachnoid hemorrhage in the course of hyperacute meningococcic sepsis]. *G Mal Infett Parassit*, **23**, 142-143.

Moncada, S. & Higgs, E.A. (1991) Endogenous nitric oxide: physiology, pathology and clinical relevance. *Eur J Clin Invest*, **21**, 361-374.

Moore, K.L., Andreoli, S.P., Esmon, N.L., Esmon, C.T. & Bang, N.U. (1987) Endotoxin enhances tissue factor and suppresses thrombomodulin expression of human vascular endothelium in vitro. *J Clin Invest*, **79**, 124-130.

Murakami, K., Okajima, K., Uchiba, M., Johno, M., Nakagaki, T., Okabe, H. & Takatsuki, K. (1996) Activated protein C attenuates endotoxin-induced pulmonary vascular injury by inhibiting activated leukocytes in rats. *Blood*, **87**, 642-647.

Murakami, K., Okajima, K., Uchiba, M., Johno, M., Nakagaki, T., Okabe, H. & Takatsuki, K. (1997) Activated protein C prevents LPS-induced pulmonary vascular injury by inhibiting cytokine production. *Am J Physiol*, **272**, L197-L202

Naldini, A., Carney, D.H., Bocci, V., Klimpel, K.D., Asuncion, M., Soares, L.E. & Klimpel, G.R. (1993) Thrombin enhances T cell proliferative responses and cytokine production. *Cell Immunol*, **147**, 367-377.

Osterud, B. & Flaegstad, T. (1983) Increased tissue thromboplastin activity in monocytes of patients with meningococcal infection: related to an unfavourable prognosis. *Thromb Haemost*, **49**, 5-7.

Pabinger, I., Allaart, C.F., Hermans, J., Briet, E. & Bertina, R.M. (1992) Hereditary protein C-deficiency: laboratory values in transmitters and guidelines for the diagnostic procedure. Report on a study of the SSC Subcommittee on Protein C and Protein S. Protein C Transmitter Study Group. *Thromb Haemost*, **68**, 470-474.

Patracchini P, Aiello V, Palazzi P, Calzolari E, and Bernardi F. (1989) Sublocalisation of the human protein C gene on chromosome 2q13-14. *Human Genetics,* **81,** 191-195.

Powars, D., Larsen, R., Johnson, J., Hulbert, T., Sun, T., Patch, M.J., Francis, R. & Chan, L. (1993) Epidemic meningococcemia and purpura fulminans with induced protein C deficiency. *Clin Infect Dis*, **17**, 254-261.

Ramsay, M., Kaczmarski, E., Rush, M., Mallard, R., Farrington, P. & White, J. (1997) Changing patterns of case ascertainment and trends in meningococcal disease in England and Wales. *Commun Dis Rep CDR Rev*, **7**, R49-R54

Ridker, P.M., Cushman, M., Stampfer, M.J., Tracy, R.P. & Hennekens, C.H. (1997) Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men [published erratum appears in N Engl J Med 1997 Jul 31;337(5):356] [see comments]. *N Engl J Med*, **336**, 973-979.

Ridker, P.M., Hennekens, C.H., Roitman-Johnson, B., Stampfer, M.J. & Allen, J. (1998) Plasma concentration of soluble intercellular adhesion molecule 1 and risks of future myocardial infarction in apparently healthy men [see comments]. *Lancet*, **351**, 88-92.

Rintala, E., Seppala, O.P., Kotilainen, P., Pettila, V. & Rasi, V. (1998) Protein C in the treatment of coagulopathy in meningococcal disease. *Crit Care Med*, **26**, 965-968.

Rivard, G.E., David, M., Farrell, C. & Schwarz, H.P. (1995) Treatment of purpura fulminans in meningococcemia with protein C concentrate. *J Pediatr*, **126**, 646-652.

Roback, M.G., Stack, A.M., Thompson, C., Brugnara, C., Schwarz, H.P. & Saladino, R.A. (1998) Activated protein C concentrate for the treatment of meningococcal endotoxin shock in rabbits. *Shock*, **9**, 138-142.

Ross, R. (1999) Atherosclerosis--an inflammatory disease [see comments]. *N Engl J Med*, **340**, 115-126.

Sakata, Y., Loskutoff, D.J., Gladson, C.L., Hekman, C.M. & Griffin, J.H. (1986) Mechanism of protein C-dependent clot lysis: role of plasminogen activator inhibitor. *Blood*, **68**, 1218-1223.

Schiller, J.H., Storer, B.E., Witt, P.L., Alberti, D., Tombes, M.B., Arzoomanian, R., Proctor, R.A., McCarthy, D., Brown, R.R. & Voss, S.D. (1991) Biological and clinical effects of intravenous tumor necrosis factor-alpha administered three times weekly. *Cancer Res*, **51**, 1651-1658.

Scully MF, Toh CH, Hoogendoorn H, Manuel RP, Nesheim ME, Solymoss S, and Giles AR. (1993) Activation of protein C and it's distribution between it's inhibitors,

protein C inhbitor, α1-antitrypsin, α2-macroglobulin, in patients with disseminated intravascular coagulation. *Thromb Haemost,* **69,** 448-453.

Semeraro, N., Triggiani, R., Montemurro, P., Cavallo, L.G. & Colucci, M. (1993) Enhanced endothelial tissue factor but normal thrombomodulin in endotoxin-treated rabbits. *Thromb Res*, **71**, 479-486.

Sen, R. & Baltimore, D. (1986) Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. *Cell*, **47**, 921-928.

Sessler, C.N., Windsor, A.C., Schwartz, M., Watson, L., Fisher, B.J., Sugerman, H.J. & Fowler, A.A. (1995) Circulating ICAM-1 is increased in septic shock. *Am J Respir Crit Care Med*, **151**, 1420-1427.

Seymour, J.J. & Ferrera, P.C. (1998) Coincident meningitis and intracerebral hemorrhage in an unresponsive adult. *Am J Emerg Med*, **16**, 576-578.

Siebenlist, U., Franzoso, G. & Brown, K. (1994) Structure, regulation and function of NF-kappa B. *Annu Rev Cell Biol*, **10**, 405-455.

Simmonds, P., Balfe, P., Peutherer, J.F., Ludlam, C.A., Bishop, J.O. & Brown, A.J. (1990) Human immunodeficiency virus-infected individuals contain provirus in small numbers of peripheral mononuclear cells and at low copy numbers. *J Virol*, **64**, 864-872.

Sinclair, J.F., Skeoch, C.H. & Hallworth, D. (1987) Prognosis of meningococcal septicaemia [letter]. *Lancet*, **2**, 38

Smith, O.P. & White, B. (1999) Infectious purpura fulminans: diagnosis and treatment. *Br J Haematol*, **104**, 202-207.

Smith, O.P., White, B., Vaughan, D., Rafferty, M., Claffey, L., Lyons, B. & Casey, W. (1997) Use of protein-C concentrate, heparin, and haemodiafiltration in meningococcus-induced purpura fulminans [see comments]. *Lancet*, **350**, 1590-1593.

Suzuki, K., Deyashiki, Y., Nishioka, J. & Toma, K. (1989) Protein C inhibitor: structure and function. *Thromb Haemost*, **61**, 337-342.

Taylor, F., Chang, A., Ferrell, G., Mather, T., Catlett, R., Blick, K. & Esmon, C.T. (1991) C4b-binding protein exacerbates the host response to Escherichia coli. *Blood*, **78**, 357-363.

Taylor, F.B.J. (1994) Studies on the inflammatory-coagulant axis in the baboon response to E. coli: regulatory roles of proteins C, S, C4bBP and of inhibitors of tissue factor. *Prog Clin Biol Res*, **388**, 175-194.

Taylor, F.B.J., Chang, A., Esmon, C.T., D'Angelo, A., Vigano-D'Angelo, S. & Blick, K.E. (1987) Protein C prevents the coagulopathic and lethal effects of Escherichia coli infusion in the baboon. *J Clin Invest*, **79**, 918-925.

Thomson, A.P., Sills, J.A. & Hart, C.A. (1991) Validation of the Glasgow Meningococcal Septicemia Prognostic Score: a 10-year retrospective survey [see comments]. *Crit Care Med*, **19**, 26-30.

Tracey, K.J., Fong, Y., Hesse, D.G., Manogue, K.R., Lee, A.T., Kuo, G.C., Lowry, S.F. & Cerami, A. (1987) Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature*, **330**, 662-664.

Tremoli, E., Camera, M., Toschi, V. & Colli, S. (1999) Tissue factor in atherosclerosis. *Atherosclerosis*, **144**, 273-283.

van der Poll, T., Levi, M., Hack, C.E., ten Cate, H., van Deventer, S.J., Eerenberg, A.J., de Groot, E.R., Jansen, J., Gallati, H. & Buller, H.R. (1994) Elimination of interleukin 6 attenuates coagulation activation in experimental endotoxemia in chimpanzees. *J Exp Med*, **179**, 1253-1259.

van Deuren, M., van Dijke, B.J., Koopman, R.J., Horrevorts, A.M., Meis, J.F., Santman, F.W. & van der Meer, J.W. (1993) Rapid diagnosis of acute meningococcal infections by needle aspiration or biopsy of skin lesions [see comments]. *BMJ*, **306**, 1229-1232.

Vieussaux, M. (1805) Memoire sur le maladie qui a regne a Geneve au primtemps de 1805. *Journal de Medicine et Chirurgie Pharmacologique*, **11**, 163

Waage, A., Brandtzaeg, P., Espevik, T. & Halstensen, A. (1991) Current understanding of the pathogenesis of gram-negative shock. *Infect Dis Clin North Am*, **5**, 781-791.

Waage, A., Halstensen, A. & Espevik, T. (1987) Association between tumour necrosis factor in serum and fatal outcome in patients with meningococcal disease. *Lancet*, **1**, 355-357.

Waage, A. & Steinshamn, S. (1993) Cytokine mediators of septic infections in the normal and granulocytopenic host. *Eur J Haematol*, **50**, 243-249.

Westendorp, R.G., Hottenga, J.J. & Slagboom, P.E. (1999) Variation in plasminogen-activator-inhibitor-1 gene and risk of meningococcal septic shock. *Lancet*, **354**, 561-563.

Westendorp, R.G., Langermans, J.A., Huizinga, T.W., Verweij, C.L. & Sturk, A. (1997) Genetic influence on cytokine production in meningococcal disease [letter; comment]. *Lancet*, **349**, 1912-1913.

Wolkow, P.P. (1998) Involvement and dual effects of nitric oxide in septic shock. *Inflamm Res*, **47** , 152-166.

Yamamoto, K., Shimokawa, T., Kojima, T., Loskutoff, D.J. & Saito, H. (1999) Regulation of murine protein C gene expression in vivo: effects of tumor necrosis factor-alpha, interleukin-1, and transforming growth factor-beta. *Thromb Haemost*, **82**, 1297-1301.

Yao, J., Mackman, N., Edgington, T.S. & Fan, S.T. (1997) Lipopolysaccharide induction of the tumor necrosis factor-alpha promoter in human monocytic cells. Regulation by Egr-1, c-Jun, and NF- kappaB transcription factors. *J Biol Chem*, **272**, 17795-17801.

Zuckerman, S.H., Evans, G.F. & Guthrie, L. (1991) Transcriptional and post-transcriptional mechanisms involved in the differential expression of LPS-induced IL-1 and TNF mRNA. *Immunology*, **73**, 460-465.

PUBLICATIONS RELATED TO THESIS

1. Successful use of Protein C, Heparin and CVVHDF in meningococcal infection.
   Smith O.P, **White B**, Vaughan D, Rafferty M, W Casey.
   Lancet 1997; 350: 1590-1593.

2. Protein C replacement therapy in meningococcal infection
   Smith OP, Mc Mahon C and **White B**
   Lancet 1998 351: 988-89 **(letter)**

3. Infectious purpura fulminans; diagnosis and treatment
   Smith OP and **White B**
   British journal of Haematology 1999 Feb; 104(2):202-207

4. Infectious purpura fulminans: Caution needed in the use of Protein C
   Smith OP and **White B**
   British Journal of Haematology (letter) 1999; 106: 253

5. Activated protein C inhibits MIF-1α and TNFα in THP-1 cells
   Schmidt M, Murphy C, **White B** Smith OP
   European Cytokine Network *in press*

6. An open label study on the use of PC replacement therapy in purpura fulminans associated with severe meningococcaemia
   White B, Livingstone W, Murphy C, Hodgson A, Rafferty M and Smith OP
   Blood *Accepted for publication*

7. Molecular basis for the anti-inflammatory properties of activated Protein C.
   White B, Schmidt M, Murphy C, Lawler M, O Toole D, Smith OP
   British Journal of Haematology 2000;110:130-134

8. Molecular insights into the anti-inflammatory properties of activated protein C
   White B, Livinstone W, Murphy C, O'Neill L, Lawler M, and Smith OP.
   British Journal of Haematology 2000;108: (suppl) 48

9. Anti-inflammatory properties of activated protein C.
   White B, Livinstone W, Murphy C, O'Neill L, Lawler M, and Smith OP.
   Blood 1999;94: (suppl 1) 2037

10. The role of protein C replacment therapy in meningococcaemia
    White B, Mc Mahon C and Smith OP
    British Journal of Haematology 1998; 101 :(suppl) 176

11. Successful use of protein C replacement therapy in meningococcal septicemia
    White B, Mc Mahon C and Smith OP
    Blood 1998; 92 (10); (suppl) 2765

12. Protein C replacement therapy in meningococcal infection.
White B and Smith OP.
Thrombosis and Haemostasis 1997; 77: (suppl) 1709.

13. Protein C replacement therapy in meningococcal infection.
White B, Casey W , Vaughan D and Smith OP.
Blood 1997; 90:10 (suppl 1) 2092.

14. The role of protein C replacment therapy in meningococcaemia
White B and Smith OP
The Irish Journal of Medical Science Vol 167, Supplement 5 pp18: 1997.

PRIZES AWARDED FOR RESEARCH RELATED TO THESIS

Sir Patrick Dun's Gold Medal, Irish National Scientific Meeting 1997

Registrars Gold Medal, Irish National Scientific Meeting 1999