ISOLATION AND CHARACTERISATION OF CYTOKINE-MODULATING PROTEINS FROM BACTERIA IMPLICATED IN INFECTIVE ENDOCARDITIS

Thesis submitted by

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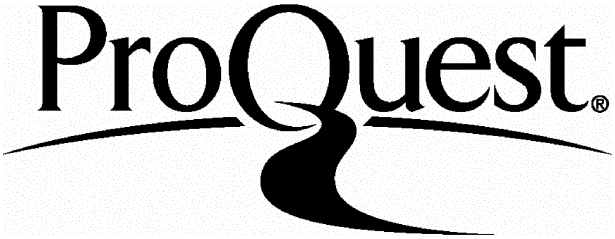

Dedicated to Michael Stevens

and in memory of my grandfather,

Dr William H Banks

ABSTRACT

The term infective endocarditis describes the inflammation of the heart caused by the colonisation of cardiac tissues, generally the valves, by microorganisms. Relatively little is known about the components of the causal bacteria that are responsible for perpetuating the inflammatory state. Soluble, exported material (EM) produced by *Streptococcus sanguis*, one of the most common causal agents of infective endocarditis, was investigated for a potential role in modulating inflammatory cytokine responses.

Gentle saline extraction of blood agar-grown *S. sanguis* yielded proteinaceous molecules with the ability to induce inflammatory cytokine release (IL-1β, IL-6, IL-8 and TNFα) from human immune cells. A role for CD14 in this activity was demonstrated. Use of a commercial cDNA array revealed that numerous pro-inflammatory cytokines were upregulated in PBMC in response to this material, including IL-1β, IL-8, IL-17 and G-CSF. Upregulation of cytokines purported to inhibit inflammation (e.g. IL-4, IL-10 and IL-1ra) was not observed, suggesting that the protein(s) selectively modulate the inflammatory cytokine network in a damaging fashion. Two proteins with IL-6-inducing activity were isolated: one of approximately 190kDa, the other a 23kDa protein that was highly homologous to the manganese-dependent superoxide dismutases of *S. mutans* and *S. pneumoniae*.

Exported material extracted from *S. sanguis* grown in the absence of blood was able to inhibit LPS-induced IL-6 production in PBMC, an activity that was not observed in EM from blood-grown bacteria. The active factor was partially purified from culture supernatant, and contained 34 and 39kDa proteins, and two proteins of greater than 66kDa, which may have been responsible for the activity.

In conclusion, *S. sanguis* secretes material that is able to modulate inflammatory cytokine networks, shifting the balance towards a damaging inflammatory response in the presence of blood, but expressing cytokine-inhibitory activity in the absence of blood. Some progress has been made towards isolating the components responsible for these activities.

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ABBREVIATIONS

ADP	Adenosine diphosphate
APS	Ammonium persulphate
ARDS	Acute respiratory distress syndrome
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CAPS	3-[cyclohexylamino]-1-propane sulfonic acid
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propane sulphonate
DTT	Dithiothreitol
ELISA	Enzyme-linked immunosorbent assay
EM	Exported material
FCS	Foetal calf serum
fMLP	formyl-Met-Leu-Phe
G-CSF	Granulocyte-colony stimulating factor
HOCl	Hyperchlorous acid
HPLC	High pressure liquid chromatography
HRP	Horse radish peroxide
HUVEC	Human umbilical vein endothelial cells
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
kDa	Kilodaltons
LAL	*Limulus* amoebocyte lysate
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
mAb	Monoclonal antibody
MIC	Minimum inhibitory concentration
MIP	Macrophage inflammatory protein
MOPS	3-Morpholinopropanesulfonic acid
mRNA	Messenger ribonucleic acid
NBTV	Non-bacterial thrombotic vegetation

OPD	1,2-phenylenediamine dihydrochloride orthophenylene diamine
PAAP	Platelet aggregation-associated protein
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PG	Peptidoglycan
PMP	Platelet microbicidal protein
PVDF	Polyvinylidene difluoride
PVE	Prosthetic valve endocarditis
RANTES	Regulated upon activation normal T-cell expressed and secreted
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
sHA	Saliva-coated hydroxyapatite
sIL-6R	Soluble IL-6 receptor
SOD	Superoxide dismutase
TEMED	N,N,N',N'-tetramethylethylenediamine
TF	Tissue factor
TFA	Tissue factor activity
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TNF-R	TNF receptor
tRNA	Transfer ribonucleic acid
TSBY	Trypticase Soya Broth with Yeast extract
WCB	Wilkins Chalgren broth
WCA	Wilkins Chalgren agar

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And finally to Mike, who gave me the confidence and the inspiration to think. My verbal eloquence fails me...

DECLARATION

This thesis is the result of my own original investigations, except where otherwise stated. The electron microscopy work described in Chapter 3 was performed by Ms. Nicky Morden of the EM Unit at the Eastman Dental Institute. The maintenance of the mouse hybridoma cell line and production and purification of the 60bca monoclonal antibody was carried out by Dr. Jo Lewthwaite of the Cellular Microbiology Research Group at the Eastman Dental Institute. The synthesis of the cDNA probes for the cDNA work was carried out by Dr Peter Tabona of the Cellular Microbiology Research Group at the Eastman Dental Institute. The protein sequencing work mentioned in Chapter 5 was carried out by Angela Paul at the Institute of Cancer Research, London.

PUBLICATIONS AS A RESULT OF THIS THESIS

ABSTRACTS

Crean SJ., Banks J., ReddiVA., Nair SP., Wilson M., Harris M., Henderson B. Pro-inflammatory activity of surface associated material from two commonly isolated Gram-positive cocci. J. Dent. Res (1998); 77:781

CHAPTER 1

INTRODUCTION

1.1 Infective endocarditis

Infective endocarditis is a life-threatening disease caused by the growth of micro-organisms on the endothelial surfaces, generally the valves, of the heart. The disease is characterised by the development of thrombotic vegetations, consisting of bacteria, fibrin and platelets, leading to a chronic, inflammatory condition. Once, almost without exception, a fatal disease, the introduction of antimicrobial chemotherapy reduced the mortality rate dramatically. However, despite the use of antibiotics, recurrent episodes of disease are not uncommon and mortality is still unacceptably high at between 12 and 25% (Dyson *et al.* 1999). The failure of antimicrobial therapy is partly due to the nature of the infection; the bacteria are to a certain extent protected by the dense thrombotic vegetation within which they are encased on the heart's surface. In addition, there is the disturbing problem of the continued emergence of antibiotic resistant strains of bacteria, particularly among the most common causal agents of infective endocarditis: the streptococci and the staphylococci (Perry *et al.* 1999). Even in cases where the infecting organism is successfully eradicated, the heart is left with permanent lesions as a result of inflammation and repair processes, a situation that not only compromises the functional properties of the tissue, but predisposes to further episodes of infective endocarditis. Since antibiotic therapy for infective endocarditis is often necessarily prolonged, adjunctive therapies may prove useful in reducing irreversible inflammation-induced damage to heart tissues. The elucidation of the infectious disease processes involved in infective endocarditis is a crucial step towards designing novel drugs or alternative treatment strategies.

1.1.1 Incidence

Infective endocarditis is a relatively uncommon disease. Since it is not notifiable, the exact incidence is not known. Extrapolation of the data obtained in a regional study in 1985 (Skehan *et al.* 1985) predicted that approximately 1000 new cases occur in

England and Wales each year (Young, 1987), and the incidence has not changed markedly in recent years (Hoesley and Cobbs, 1999). Although the numbers are relatively low, the mortality rate for this disease is still unacceptably high, despite the use of antibiotics. In the 1930's, before antibiotics became available, there were over 1,200 deaths per year in England and Wales attributable to infective endocarditis; by 1953, following the introduction of antibiotics, this figure had decreased to 339, and a further decrease occurred as emergency valve replacement became possible (Bayliss *et al.* 1986). The data from Skehan's study (1985) predicted just over 200 deaths per year, a figure that is in agreement with the actual number of registered endocarditis-attributable deaths in the year studied (Young, 1987). In a more recent study of 128 microbiologically-positive cases of infective endocarditis between 1987 and 1996, the mortality rates for native valve and prosthetic valve endocarditis were 12.3 and 24.5% respectively (Dyson *et al.* 1999).

1.1.2 Development of infective endocarditis

The series of events leading to the development of infective endocarditis is illustrated in figure 1.1. Although the disease can develop in healthy individuals there is usually some form of pre-existing damage to the endothelium of the valve, which may be caused by a number of factors. In the past, rheumatic fever was a relatively common disease that often left survivors with irregularities on the surfaces of heart valves, predisposing them to infective endocarditis (O'Sullivan and Cobbs, 1998). For the same reason, previous episodes of endocarditis also increase the risk of developing the disease, as healed endocardial lesions remain on the tissue surface. The incidence of cardiac valve replacement is increasing (ironically this is sometimes to replace an infected native valve), and this can be complicated by the development of prosthetic valve endocarditis (PVE) (Hyde *et al.* 1998). In fact, prosthetic heart valves were found to be the most important risk factor in developing infective endocarditis in a two year survey of 582 episodes (Bayliss *et al.* 1986). Thereplacement of a heart valve inevitably leads to small areas of endothelial damage, particularly around the sewing cuff (where the prosthetic device is attached to the tissue) (Horstkotte *et al.* 1995).

Irrespective of cause, altered endothelial surfaces develop small thrombi from blood components (non-bacterial thrombotic vegetations - "NBTV") and this is one of the key events in the development of infective endocarditis. This is the basis for the animal models of infective endocarditis whereby a catheter inserted into the heart leads to the development of NBTV which are then colonised by bacteria (Garrison and Freedman, 1970; Durack and Beeson, 1972b). The other pivotal event is a transient bacteraemia. Historically it was thought that most cases of infective endocarditis could be traced back to a dental procedure which had released bacteria into the bloodstream. However, a survey by the Royal College of Physicians Research Unit suggested that as few as 12% of 544 episodes were due to dental procedures (Bayliss *et al.* 1983), and bacteraemia often occurs without obvious cause: in fact streptococcal bacteraemia can result from toothbrushing (Durack, 1995). In general, the organism isolated from the vegetation reflects how the bacteraemia arose. For example intravenous drug users tend to present with *Staphylococcus aureus* endocarditis, an organism commonly found on the skin.

Bacteria can attach directly to the heart tissues or to deposited platelets and fibrin, and a number of adhesins from endocarditis-causing bacteria have been identified (see section 1.2.4.1.). The vegetation develops as the bacteria multiply and platelets aggregate. More fibrin is deposited leaving the bacteria encased in a solid platelet-fibrin mass.

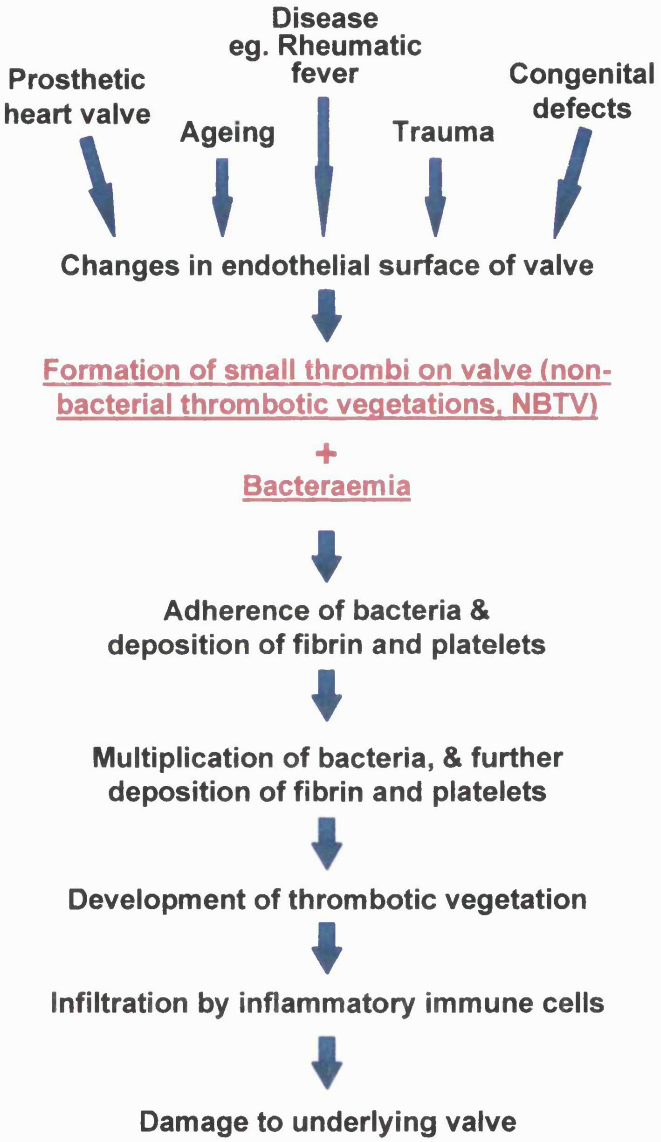

Figure 1.1 Flow diagram showing the events in the development of infective endocarditis.

1.1.3 Morphology of the endocardial vegetation

The vegetations can be several centimetres in diameter and there may be multiple growths. They have a warty, "cauliflower-like" appearance and are readily fragmented. Figure 1.2. shows a human heart with endocardial vegetations caused by "Viridans streptococci" (McLay *et al.* 1997). Histological staining reveals irregular masses of fibrin, platelets and bacteria (Figure 1.3.) The surface of the vegetation may be covered with mononuclear cells (Curran, 1985), but there are few immune cells present in the vegetation itself. Underneath, the valve tissue is heavily inflamed and vascularised with a wide range of immune cells present, particularly tissue macrophages and polymorphonuclear leukocytes (Cawson *et al.* 1982; Virmani *et al.* 1996; Franklin, 1998). By growing inside the platelet-fibrin matrix, the colonising bacteria are to a certain extent protected from both circulating immune cells and those localised in the valve tissues.

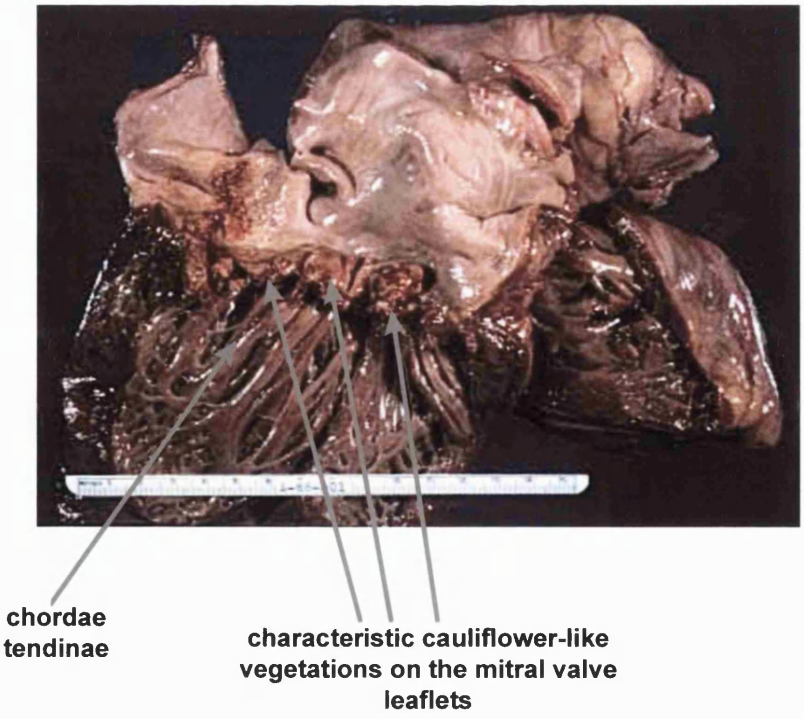

Figure 1.2 Photograph of a heart from a patient with infective endocarditis. Adapted from McLay *et al.* (1997).

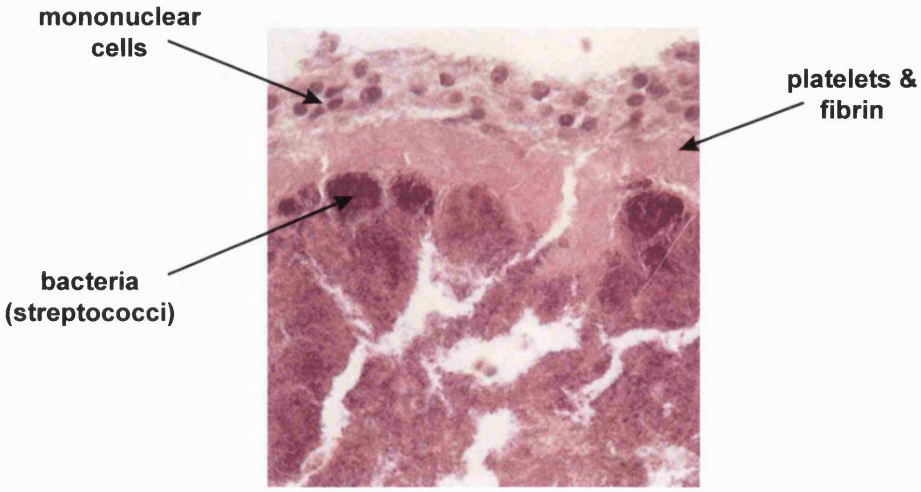

Figure 1.3 Histological stain of a section through an infected endocardial vegetation. Adapted from Curran (1985).

1.1.4 Clinical course

The first symptoms of infective endocarditis are usually fever, malaise, loss of appetite and weight. Patients may also suffer from anaemia, splenomegaly and immune complex formation which may lead to glomerulonephritis, vasculitis or arthritis. These symptoms are mainly due to the constant shedding of bacteria from the vegetation into the bloodstream (Freedman, 1987). Fragments from the infected vegetation may break free, circulate in the bloodstream and lodge in the arteries supplying other organs, commonly the kidneys, spleen and intestine. If this happens in the blood supply to the brain, it can result in a stroke (Hart et al. 1990). Lodged peripheral emboli may induce a local infection and, if weakened enough, the blood vessel may rupture causing massive haemorrhage. The heart valves themselves become vascularised and thickened, and infiltrated with leukocytes (Franklin, 1992). Valvular function is thus impaired and abnormalities in cardiac conductivity may develop. The valvular tissue is progressively destroyed, and heart failure will eventually result. Congestive heart failure is the most common cause of death in infective endocarditis (Erbel et al. 1995), and is particularly associated with *Staph. aureus* infection. In a study of 119 cases of *Staph. aureus* infective endocarditis, 61% developed congestive heart failure (Esperson

and Frimodt-Moller, 1986). Without treatment, infective endocarditis is fatal, as illustrated by the fact that in the pre-antibiotic era, incidence equalled mortality (Bayliss *et al.* 1986).

1.1.5 Treatment

In the treatment of infective endocarditis the most commonly used antibiotics have been those that target the bacterial cell wall (penicillins, cephalosporins and vancomycin) (Korzeniowski, 1998), and specific guidelines for the treatment of streptococcal, enterococcal and staphylococcal endocarditis are laid out in a report by the Working Party of the British Society for Antimicrobial Chemotherapy (Anonymous1998). As a first line of treatment, penicillin has been used with relative success against Gram-positive organisms, being particularly effective when administered in combination with aminoglycosides, usually gentamicin, with the glycopeptides vancomycin and teicoplanin providing alternatives for patients who are allergic to penicillin. However, failure to completely eradicate the infecting bacteria is not uncommon, the result being recurrent episodes of disease. The inefficacy of antimicrobial chemotherapy is due in part to the nature of the infection; the bacteria grow within a dense, fibrous mass of platelets and fibrin, and this vegetation can be considered to be a biofilm by Costerton's definition: "...matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces..." (Costerton *et al.* 1995). Biofilms are notoriously refractive to antibiotic sterilisation, and the four main hypotheses proposed to explain this phenomenon were described by Brown and Gilbert (Brown and Gilbert, 1993). The first is that the glycocalyx (polysaccharide that is produced by biofilm-colonising bacteria) inhibits the access of antibiotics to the bacterial cells. Although the matrix of an endocardial vegetation is mostly made up of fibrin and platelets rather than just glycocalyx, it is feasible that antimicrobial access might be inhibited in a similar way. The second hypothesis is that chemically-reactive antibiotics are quenched by reactions with surface regions of the matrix and outlying cells before they reach the more deeply embedded colonies. The third is that the matrix causes nutrient gradients to form within the biofilm, and bacteria in regions of limited nutrient availability grow more

slowly, with some entering a relatively dormant state. Changes in growth rate are accompanied by changes in the composition of the cell envelope and this in turn influences susceptibility to drugs. In particular, slow-growing cells are recalcitrant to the effects of antibiotics (Brown *et al.* 1990; Gilbert *et al.* 1990). The fourth hypothesis is that the attachment of bacteria to surfaces triggers the expression of a series of genes for a biofilm growth phenotype, which also affect antimicrobial susceptibility. It has been shown that *Pseudomonas aeruginosa* controls the transition from planktonic to biofilm growth by a sigma factor, similar to that used by Gram-positive bacteria to control sporulation (Martin *et al.* 1994; Deretic *et al.* 1994). Recently, Loo and colleagues have used transposon mutagenesis to pinpoint genes required by *S. gordonii* for growth as a biofilm (Loo *et al.* 2000). These adherence-induced phenotypic changes, at least in some species, may include an increased resistance to antibiotics. The failure of antibiotics to sterilise biofilms is probably due to a combination of these factors.

In addition to the inherent resistance of bacteria to antibiotics, there is also the growing problem of antibiotic resistant strains of bacteria, particularly with the staphylococci, but also now among the streptococci (Doern *et al.* 1996; Wisplinghoff *et al.* 1999). The emergence of multiple drug resistance is of particular concern (de Azavedo *et al.* 1999; Poutanen *et al.* 1999). Van der Meer and colleagues in the Netherlands found that approximately 11% of cases of viridans streptococcal endocarditis were due to antibiotic resistant strains (van der Meer *et al.* 1991). In a more recent study of 100 isolates from cases of infective endocarditis, 78% of streptococcal isolates were found to be tolerant to teicoplanin, while 57% were tolerant to vancomycin (Perry *et al.* 1999). The results for enterococcal isolates were even more disturbing, with virtually all isolates exhibiting resistance to both antibiotics. Since glycopeptides such as teicoplanin and vancomycin are commonly used to treat infective endocarditis, the emergence of resistance to these antibiotics is worrying.

It is possible that the treatment of other unrelated systemic diseases with antibiotics may favour the emergence of resistant bacteria by selective pressure. To investigate the

possibility that this might occur *in vivo*, Herzberg and colleagues investigated the prevalence of antibiotic-resistant *S. sanguis* isolates in dental plaque from children that had been treated with antibiotics in the previous month (Erickson and Herzberg, 1999). All had completed a ten day course of amoxicillin, trimethoprim-sulfamethoxazole or erythromycin-sulfisoxazole, while the control group had received no antibiotics in the previous 24 months. None of the *S. sanguis* isolates from the control group was resistant to penicillin, amoxicillin, trimethoprim-sulfamethoxazole or erythromycin. In contrast, 60% of the isolates from the experimental group were resistant to one or more antibiotic, and 28% were resistant to two or more unrelated antibiotics. There was no difference in the frequency of isolation of *S. sanguis* between the two groups. Cystic fibrosis patients, who frequently undergo antibiotic treatment, have also been suggested to represent a potential reservoir of resistant viridans streptococcal isolates (Alvarez *et al.* 1998).

Whether the disease involves a native or a prosthetic valve, a fast and accurate microbiological diagnosis must be achieved to establish the most appropriate antibiotic therapy, and this should include evaluation of the minimum inhibitory concentration (MIC). Even with susceptible bacterial strains, eradication is by no means certain. In prosthetic valve endocarditis (PVE), where the minimum bactericidal concentration (MBC) is greater than 4μg/ml, sterilisation of the vegetation by antibiotics is unlikely, and as a general rule, the larger the vegetation, the more likely the failure of the antibiotics (Horstkotte *et al.* 1995) Where antibiotics fail, the only solution is surgical intervention and valve replacement, a solution that brings with it the further risk of developing prosthetic valve endocarditis.

1.1.6 Endocarditis chemoprophylaxis

Virtually all invasive medical and dental procedures can result in bacteraemia (Durack, 1995). Such bacteraemias are short-lived, with those resulting from dental extractions lasting less than 10 minutes (Roberts *et al.* 1992). However, in animal models it can take just minutes for bacteria introduced into the bloodstream to adhere to the endocardium (Durack and Beeson, 1972a; Durack, 1975). In patients with known

underlying cardiac abnormalities undergoing invasive procedures, the Working Party of the British Society for Antimicrobial Chemotherapy recommend antibiotic prophylaxis (Anonymous1990), although these guidelines are not always followed (Martin *et al.* 1997). The categories of dental or medical procedure implicated for chemoprophylaxis are shown in table 1.1, and the patient risk categories are shown in table 1.2.

Table 1.1. Procedures for which endocarditis prophylaxis is recommended

Dental extractions, scaling, or periodontal surgery
Surgery or instrumentation of the upper respiratory tract
Genito-urinary surgery or instrumentation
Obstetric and gynaecological procedures
Gastrointestinal procedures

Table 1.2. Patient risk categories for antibiotic prophylaxis

Chronic rheumatic heart disease or history of rheumatic fever
Congenital heart disease
Prosthetic heart valves
Mitral or aortic incompetence
Previous history of infective endocarditis
Hypertrophic cardiomyopathy
Heart murmurs
Systolic murmurs, especially in the elderly
Intravenous drug abusers

The protective efficacy of endocarditis chemoprophylaxis is still uncertain. Although animal models have demonstrated that antibiotics could effectively eradicate streptococci if administered early enough (Durack, 1995; Perdikaris *et al.* 1997), there are reports of patients who have developed infective endocarditis, despite appropriate preoperative antibiotics being given (O'Sullivan *et al.* 1996; Martin *et al.* 1997). In a case-control study of 438 patients on the prevention of endocarditis, the authors concluded that prophylaxis was not effective (van der Meer *et al.* 1992); the only other

documented clinical study reported a protective efficacy of 91% for prophylaxis but examined only 8 cases of infective endocarditis (Imperiale and Horwitz, 1990). It has been suggested that a much smaller percentage of cases of infective endocarditis result from dental procedure-induced bacteraemia than was previously thought (Bayliss *et al.* 1983). In agreement with this data are the findings of Lacassin and colleagues who reported that although endocarditis prophylaxis may be important for some invasive procedures, dental procedures did not represent a risk factor (Lacassin *et al.* 1995). Chemoprophylaxis for dental procedures is therefore unlikely to greatly reduce the number of cases of infective endocarditis, and its use is increasingly questioned (Strom *et al.* 1998; Tong and Rothwell, 2000). However, due to the life-threatening nature of the disease, the use of antibiotic prophylaxis for "at risk" patients will undoubtedly continue, even in the light of increasing antibiotic resistance among bacteria.

1.1.7 Microorganisms implicated in infective endocarditis

It would appear that almost any microorganism can cause infective endocarditis (Weinstein and Brusch, 1996). However, particular bacteria are isolated from vegetations with much greater frequency than others, namely streptococci and staphylococci. The published results of many surveys are limited in the extent to which isolates have been identified; a single value is often presented for "viridans streptococci" or "coagulase-negative staphylococci". In a survey carried out by the Royal College of Physicians (RCP) Research Unit (Bayliss *et al.* 1986) streptococci were isolated from 63% of cases and staphylococci from 20%, with *Staph. aureus* accounting for more than 50% of the staphylococcal cases. Similar results were published by Delahaye and colleagues who carried out a one year survey in France, the results of which are shown in Table 1.3. (Delahaye *et al.* 1995). The data collected was from more than 500 patients with infective endocarditis, and a causal microorganism was identified in 92% of cases. A more recent study revealed that the viridans streptococci remain the predominant organisms in infective endocarditis (Dyson *et al.* 1999). The RCP study reported *S. sanguis* as being the most frequently isolated streptococcal species, although this was prior to significant changes in the

classification of the oral streptococci (Kilian *et al.* 1989; Whiley *et al.* 1990; Handley *et al.* 1991; Whiley and Beighton, 1991). To determine which of the newly-defined species of oral streptococci were the most important causal agents, Douglas and colleagues examined 47 strains of oral streptococci from cases of infective endocarditis (Douglas *et al.* 1993). *S. sanguis* was the most frequently isolated organism, accounting for 32%, closely followed by *S. oralis* (30%). Approximately 13% of isolates were identified as *S. gordonii.*

Table 1.3. Microbiology of isolates from 575 cases of native valve endocarditis.

Streptococci (total)	**58%**
Viridans streptococci	(27%)
Other streptococci	(21%)
Staphylococci (total)	**23%**
Staph. aureus	(18%)
CNS	(4%)
Other staphylococci	(1%)
Other	**11%**
Culture negative	**8%**

CNS: coagulase-negative staphylococci. Adapted from Delahaye et al (1995).

The enterococci also constitute a significant cause of infective endocarditis, being responsible for between 5 and 15% of cases (Murray, 1990) and are of particular concern due to the high level of antibiotic resistance among isolates from cases of infective endocarditis (Johnson *et al.* 1998).

Prosthetic valve endocarditis (PVE) is defined as an infection on either parts of a mechanical or biological valve substitute, or on reconstructed native heart valves, and the microbiology is significantly different to that of native valve endocarditis (Horstkotte *et al.* 1995). As is seen in other device-related infections (Huebner and Goldmann, 1999), *Staph. epidermidis* is the predominant coloniser. Table 1.4. shows

the microbes most frequently isolated from cases of PVE, but it should be noted that there are few organisms that have not been linked to at least one case of PVE.

Table 1.4. Microbiology of isolates from 140 cases of PVE.

	Early PVE*	Late PVE
Staph. epidermidis	28.1%	20.4%
Staph. aureus	18.8%	19.4%
HACEK	18.1%	7.4%
Fungi	9.4%	5.6%
Enterococci	6.3%	16.7%
Viridans streptococci	3.1%	15.7%

Early PVE is defined as occurring within 60 days of surgery. HACEK microorganisms include *Haemophilus aphrophilus/parainfluenzae, Actinobacillus actinomycetemcomitans, Cardiobacterium hominis, Eikenella corrodens and Kingella kingae.* Adapted from Horstkotte et al (1995).

Infective endocarditis is a significant cause of death in intravenous drug abusers, and, like PVE, the organisms most frequently isolated are different from those isolated from other patients. A cohort study of 125 intravenous drug users with infective endocarditis revealed that 66% of cases were due to staphylococci whilst only 26% were due to streptococci, possibly reflecting entry of microbes from the skin (Mathew *et al.* 1995).

1.2 The oral streptococci

1.2.1 Taxonomy

Despite the effort that has gone into the classification of the oral streptococci in the last century, textbooks, and even some journal publications, still use the terms "oral streptococci" and "viridans streptococci" interchangeably. This, inevitably, leads to confusion, since alpha-haemolysis (the greening reaction on blood agar after which the "viridans streptococci" were named, from the latin "*viridis*", meaning green), is not restricted to oral streptococci, and some oral streptococci exhibit beta-haemolysis. The confused classification of the oral streptococci was greatly aided by the work of Kilian

and colleagues, who subjected 151 strains to a comprehensive range of biochemical and physiological tests, as well as serological reactions (Kilian *et al.* 1989). Since this reclassification, additions to the nomenclature have included *S. parasanguis* (Whiley *et al.* 1990), *S. crista* (Handley *et al.* 1991), *S. constellatus*, *S. intermedius* and *S. anginosus* (Whiley and Beighton, 1991) and more recently, *S. peroris* and *S. infantis* (Kawamura *et al.* 1998). Currently, there are 18 recognised species of oral streptococci, with the largest number falling in the mitis group, which includes *S. sanguis* (Whiley and Beighton, 1998).

The phylogenetic relationships among the streptococci were examined by Kawamura and colleagues by means of 16S ribosomal RNA (rRNA) sequencing (Kawamura *et al.* 1995), and from this analysis, six distinct groups were arrived at: the mutans group; the anginosus group; the mitis group; the salivarius group; the bovis group, and the pyogenic group, with several species remaining ungrouped. The first four groups constitute the oral streptococci, and the species of streptococci commonly found in the human oral cavity are shown in Table 1.5.

1.2.2 *Streptococcus sanguis*

First isolated in 1946 from the blood of a patient with endocarditis (White and Niven, 1946), *S. sanguis* owes its name to the latin word for blood - "*sanguis*". The classification of mitis group streptococci, and in particular *S. sanguis*, has undergone many changes since this first identification, and as a result, the early literature is often confusing. References to "*S. sanguis* I" and "*S. sanguis* II" appear frequently, names which were proposed by Facklam in 1977 (Facklam, 1977). "*S. sanguis* I" encompassed what are now classified as *S. sanguis* and *S. gordonii*, while "*S. sanguis* II" represented two currently recognised species: *S. oralis* and *S. mitis* (Kilian *et al.* 1989). The term "*S. mitior*", which was introduced by Colman and Williams (Colman and Williams, 1972) and was essentially equivalent to Franklam's "*S. sanguis* II", is thus also redundant. Kilian and colleagues further sub-divided *S. sanguis* into biovars 1-4, on the basis of a comprehensive scheme of biochemical tests (Kilian *et al.* 1989),

while Beighton and colleagues, using a similar range of tests, divided the same species into just three biotypes (Beighton *et al.* 1991).

On the basis of 16S rRNA sequence analysis, *S. sanguis* has the closest phylogenetic relationship to *S. parasanguis*, followed by *S. gordonii* (Kawamura *et al.* 1995).

Table 1.5. Streptococci commonly found in the human mouth. Species that are not generally found in humans have not been included. Adapted from Russell (Russell, 2000).

Species group	Species
Mitis group	*S. mitis*
	S. oralis
	S. gordonii
	S. sanguis
	S. parasanguis
	S. crista
	S. infantis
	S. peroris
Mutans group	*S. mutans*
	S. sobrinus
Salivarius group	*S. salivarius*
	S. vestibularis
Anginosus group	*S. anginosus*
	S. constellatus
	S. intermedius

1.2.2.1 Habitat

S. sanguis is a normal inhabitant of the oral cavity of humans. In a study by Frandsen and colleagues on the ecology of the oral streptococci (Frandsen *et al.* 1991), 1426 streptococcal isolates from 30 samples were identified according to the species defined in the taxonomic study of Kilian and co-workers (Kilian *et al.* 1989). Bacteriological

samples were obtained from the buccal mucosa, the dorsum of the tongue, the pharynx, subgingival plaque and mature supragingival plaque. *S. sanguis* comprised 49% of the streptococcal flora of the buccal mucosa, making it the predominant *Streptococcus* species at this location. This species was also found in mature supragingival and subgingival plaque, although only accounting for 2 and 13% of streptococci isolated from these sites respectively. No isolates of *S. sanguis* were found in samples from the tongue. The closely related *S. gordonii* showed a distinctly different pattern of colonisation, its primary habitat being mature supragingival plaque, although neither it, or *S. sanguis* were prominent members of subgingival plaque. *S. sanguis* and *S. oralis* were found to be associated with the initial phase of plaque formation in samples taken 4 hours after professional tooth-cleaning. During the sequence of events in the colonisation of clean tooth surfaces to form plaque, termed microbial succession, species such as *S. mitis*, *S. oralis* and *S. sanguis* are among the first bacteria to become established (Marsh and Martin, 1999). Frandsen and colleagues (Frandsen *et al.* 1991) also investigated the IgA1 protease activity of isolates from oral sites. Such activity was associated almost exclusively with isolates from buccal mucosa and recently cleaned tooth surfaces, and it has thus been suggested that the ability to evade salivary IgA allows particular species of streptococci to colonise oral surfaces (Cole *et al.* 1994).

S. mitis and *S. oralis* are the predominant pioneer species colonising the oral cavity of neonates (Pearce *et al.* 1995), but once tooth eruption has occurred, the oral flora diversifies, and *S. sanguis* and *S. gordonii* colonise in significant numbers. With a high affinity for binding to salivary glycoprotein pellicles, which form on tooth surfaces, and the ability to bind to other oral bacteria (Jenkinson and Lamont, 1997b), *S. sanguis* is thought to play an important role in the development of the complex microbial flora of the mouth.

1.2.2.2 Morphology and physiology

S. sanguis is a nonmotile, nonsporulating Gram-positive coccus that produces lactic acid as a sole product of fermentative metabolism. Growth is anaerobic although, since

it is not sensitive to oxygen and can grow in its presence, *S. sanguis* is considered to be an aerotolerant anaerobe. Cells grow as short chains in broth culture. When grown on blood agar, colonies are surrounded by distinct zones of greening, termed "alpha-haemolysis", and extracellular polysaccharide is produced when grown on sucrose-containing agar. The cell wall contains peptidoglycan (PG) of the Lys-Ala1-3 type, and also rhamnose- and glycerol-teichoic acids.

1.2.3 Association with disease

Despite a high profile as a coloniser of the oral cavity, *S. sanguis* is not generally associated with either dental caries or the periodontal diseases. Of the oral streptococci, *S. oralis* (Willcox *et al.* 1987) and certain strains of the "*S. milleri*" group (Drucker and Green, 1978) have been shown to have some cariogenic potential, but in the latter study strains of *S. sanguis* were reported to produce little or no caries in gnotobiotic rats. Likewise, there is little evidence of involvement of *S. sanguis* in the periodontal diseases, although a review by Gossling (Gossling, 1988) describes a number of studies implicating species of the "*S. milleri*" group in these diseases. Probably most well known for its involvement in infective endocarditis, *S. sanguis* is also increasingly recognised as one of the causative agents of "α-streptococcal shock syndrome" and adult respiratory distress syndrome (ARDS) in neutropenic patients (Bochud *et al.* 1997). The syndrome is usually restricted to leukaemia patients undergoing cytotoxic chemotherapy whose immunosuppressed state leaves them susceptible to opportunistic infection by oral streptococci.

1.2.4 Virulence factors of streptococci in infective endocarditis

1.2.4.1 Adherence

Adherence of bacteria to the heart is usually considered to be the crucial event in the development of infective endocarditis. If bacteria do not adhere, the vegetation is sterile and the course of the disease is much milder. Various components of *S. sanguis*

and other oral streptococci have been investigated for a potential role in adherence to vegetations in infective endocarditis.

In the presence of sucrose, streptococci produce soluble and insoluble glucans (the latter is called dextran) and fructans, and these exopolysaccharides have been suggested as factors which may contribute to adherence and infectivity in endocarditis (Scheld *et al.* 1978). However, this would seem unlikely, since the blood and tissues do not contain sucrose (from which dextran is synthesised). Glycocalyx, on the other hand, is produced by some strains of streptococci in the absence of sucrose, and has been shown to increase colonisation of endocardial vegetations *in vivo* (Munro and Macrina, 1993). In other strains unable to produce a glycocalyx, colonisation is unaffected (Wells *et al.* 1993). Thus the role of exopolysaccharides in adherence of streptococci to endocardial vegetations is unclear.

Adhesins for connective tissue proteins are likely to be involved in bacterial adherence in infective endocarditis. On damaged endothelium types I, IV and VI collagen may become exposed, and DNA homology with the *yadA* gene of *Yersinia pseudotuberculosis* (the product of which is a fibrillar adhesin that binds collagen) has been reported in *S. sanguis* (Liu and Herzberg, 1994). Fibronectin adsorbed from the blood may be present on exposed connective tissue and several fibronectin adhesins have been identified. These include CshA/CshB from *S. gordonii* (McNab *et al.* 1996) and their homologues found within the fibrils of *S. sanguis* (McNab *et al.* 1995).

In addition to that found in plasma, fibrinogen is released from alpha granules secreted by activated platelets. Fibrinogen may then associate with connective tissue proteins, or bind to activated endothelial cells or platelets (Manning *et al.* 1994; Nagata *et al.* 1994; Vacca-Smith *et al.* 1994). Insoluble fibrin is laid down due to the cleavage of fibrinogen by thrombin, as part of the coagulation response which occurs when blood comes into contact with damaged tissue. The products of the *fimA* gene of *S. parasanguis* have been reported to be adhesins for both fibrin and fibrinogen, with *fimA* mutants displaying reduced infectivity *in vivo* (Burnette-Curley *et al.* 1995), and *S.*

sanguis possesses a gene homologous to *fimA*, *ssaB* (Lowe *et al.* 1995). However, the importance of these proteins as adhesins is not clear as the product of *fimA* is thought to be a membrane transporter (Kolenbrander *et al.* 1994; Fenno *et al.* 1995) and reduced infectivity of *FimA* mutants *in vivo* may simply be due to decreased growth.

NBTV, to which circulating bacteria may bind, contain platelets, and a number of platelet adhesins have been identified in *S. sanguis*. Gong and colleagues (Gong *et al.* 1995a; Gong *et al.* 1995b) found that the fibrils of one strain of *S. sanguis* contained a 150 kDa platelet adhesin, a 130 kDa adhesin for saliva-coated hydroxyapatite (sHA), shown to be an analogue of the antigen I/II adhesins, and an 87 kDa adhesin which bound both platelets and sHA.

1.2.4.2 Interactions with host defences

The activation of platelets can be taken to be a defensive response on the part of the host, since thrombin-induced platelet microbicidal protein (tPMP), which is released from the alpha granules of activated platelets, has been demonstrated to be microbicidal against a broad spectrum of bloodstream pathogens (Yeaman *et al.* 1992; Yeaman *et al.* 1993; Sullam *et al.* 1993). The mechanism of action against staphylococci appears to involve the direct permeabilisation of the cytoplasmic membrane (Koo *et al.* 1997). However, some strains of streptococci possess a gene encoding PMP resistance (PMP^R) (Dankert *et al.* 1995), expression of which not only enables the bacteria to withstand PMP, but also allows them to grow inside the platelet vegetation where they can avoid attack by the many other facets of the host immune response. In the rabbit model, a PMP susceptible strain of staphylococci was demonstrated to be reduced in number in vegetations after vancomycin therapy, while the same treatment had no effect on the numbers of a strain expressing PMP resistance (Dhawan *et al.* 1999).

1.2.4.3 Platelet aggregation

The ability of bacteria to aggregate platelets has been demonstrated to increase vegetation size and mortality in a rabbit model of experimental endocarditis (Herzberg

et al. 1992). In addition, a correlation was found between the ability of streptococcal isolates to aggregate platelets *in vivo* and poor disease outcome in endocarditis patients (Greaves *et al.* 1993). The viridans streptococci appear to be particularly adept at causing platelet aggregation: a study of viridans streptococcal isolates from children with streptococcal shock and signs of intravascular coagulation by Herzberg and colleagues, demonstrated that more than 60% induced platelet aggregation *in vitro* (Herzberg and Meyer, 1996). Various mechanisms have been suggested for the aggregation of platelets by streptococci.

1.2.4.3.1 Platelet aggregation associated protein (PAAP)

Certain strains of *S. sanguis* express on their fibrils a 115kDa glycoprotein which has been shown to induce platelet aggregation. This platelet aggregation associated protein (PAAP) contains a collagen-like domain which allows *S. sanguis* to bind to platelets by what appears to be a receptor for collagen (Erickson *et al.* 1992; Erickson and Herzberg, 1993). The platelet membrane receptor has been reported to be a 170kDa signal-transducing receptor (Gong *et al.* 1995b), binding to which results in platelet activation and subsequent aggregation. Activated platelets release fibrinogen, providing more substrate for thrombin to convert to insoluble fibrin which, in infective endocarditis, adds to the vegetation. The expression of PAAP by *S. sanguis* has been reported to be induced by growth in the presence of collagen (Erickson and Herzberg, 1995) or by heat shock (Liu *et al.* 1995).

1.2.4.3.2 Interactions of complement and IgG

Much of the work on platelet aggregation by *S. sanguis* has been carried out on the type strain, NCTC 7863, a strain that has been shown to aggregate platelets from more than 90% of normal subjects (Ford *et al.* 1993). Investigations by Ford and colleagues revealed that IgG, fibrinogen and complement components were necessary for aggregation to occur (Ford *et al.* 1996a; Ford *et al.* 1997b). Assembly of the complement terminal complex SC5b-9 (sometimes called the membrane lysis complex) on the surface of *S. sanguis* was found to be a crucial step in the initiation of

aggregation (Ford *et al.* 1996a). Other workers have demonstrated that vitronectin (an adhesive glycoprotein) associates with the complement terminal complex and inhibits complete insertion of the pore-forming SC5b-9 into the bacterial membrane, and thus prevents cell lysis (Preissner and Jenne, 1991). Vitronectin may therefore promote aggregation by preventing bacterial cell lysis.

The question of which components actually bound *S. sanguis* to the platelet membrane was addressed by Ford and colleagues (Ford *et al.* 1996a; Ford *et al.* 1996b). They demonstrated that if the bacteria were first allowed to become coated with all the necessary plasma components, aggregation could be blocked by antibodies to IgG, and inhibited by anti-vitronectin antibodies, while antibodies to complement components had no effect. Aggregation was also blocked with a monoclonal antibody to the platelet receptor for IgG, FcγRII (Ford *et al.* 1997b), although whether this receptor is involved in signal transduction, or just adhesion, is not yet known. The use of monoclonal antibodies also demonstrated a requirement for the platelet glycoprotein, GPIIb-IIIa (Ford *et al.* 1993), and the receptor for vitronectin, $\alpha_v\beta_3$ (Ford *et al.* 1996b). The scheme, proposed by Ford and colleagues, by which *S. sanguis* NCTC 7863 induces platelet aggregation (Ford and Douglas, 1997a) is summarised in Figure 1.4.

Platelet activation and aggregation occur as a result of the immunological defense mechanisms that are triggered in response to this strain of *S. sanguis*. Since the extent of aggregation has been correlated with disease severity and mortality, this appears to be a classic example of the double-edged sword of the immune response.

1.2.4.4 Ecto Adenosine Triphosphatase (EctoATPase)

S. sanguis has been reported to express an EctoATPase on the cell wall which potentiates the induction of platelet aggregation *in vitro* (MacFarlane *et al.* 1994). The enzyme hydrolyses ATP (released by activated platelets) to ADP, which is a platelet agonist and would potentially amplify the aggregation response induced by *S. sanguis*.

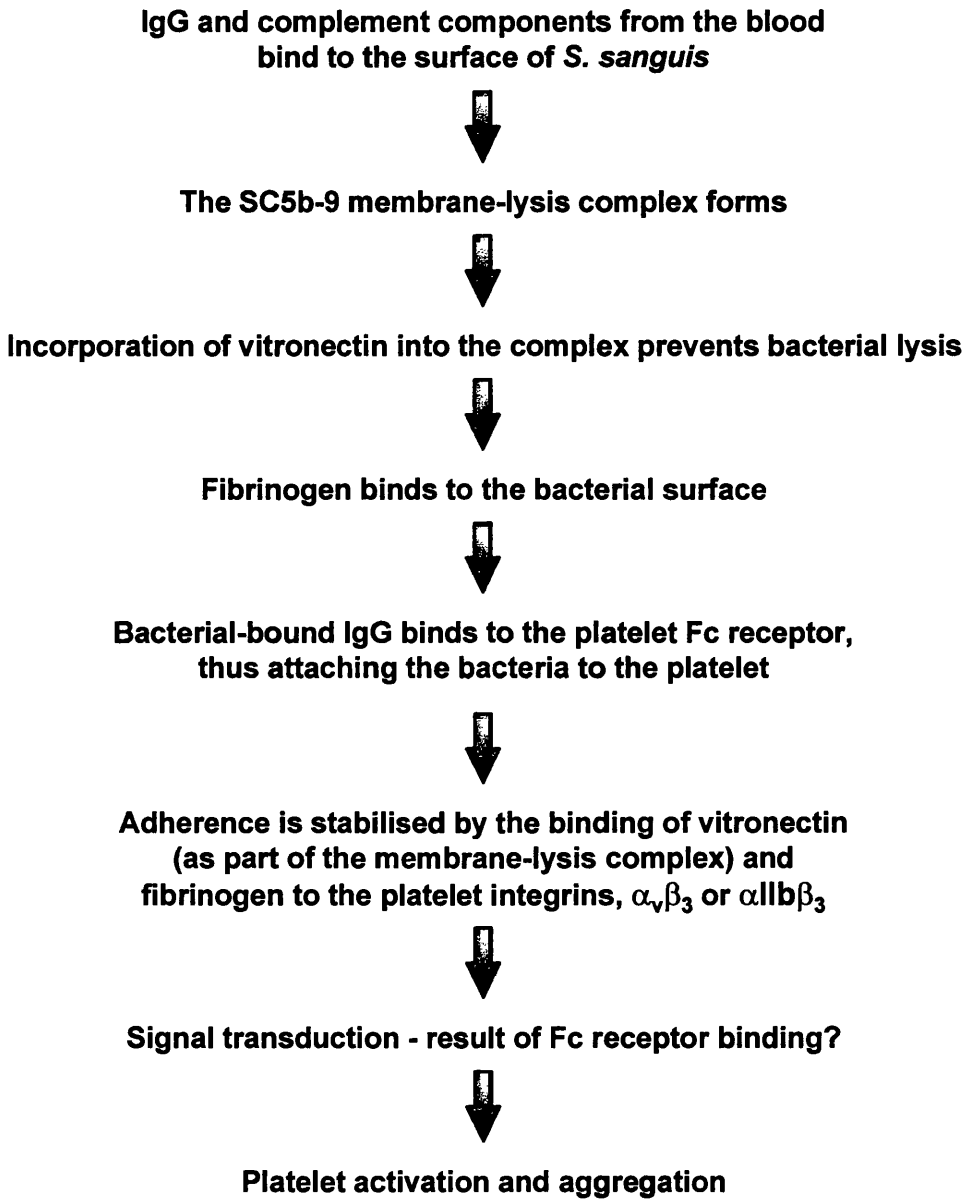

Figure 1.4 Flow chart showing the series of events proposed by Ford and colleagues for the aggregation of platelets by *S. sanguis* strain NCTC 7863.

1.2.4.5 Pro-coagulant activity

Since PAAP negative strains of *S. sanguis* still form small vegetations in experimental endocarditis (Herzberg *et al.* 1992), other mechanisms must also be involved in the generation of thrombotic vegetations. One such alternative mechanism is the promotion of coagulation and the laying down of fibrin. The extrinsic coagulation pathway has been demonstrated to be involved in infective endocarditis (Buiting *et al.* 1989), and this pathway is initiated by the binding of Factor VII to host cell-surface tissue factor (TF). Cultured endothelial cells from cardiac valves exhibited increased TF expression in response to *Staph. aureus* (Drake and Pang, 1988), IL-1 and LPS (Drake and Pang, 1989), but not to whole, viable viridans streptococci, enterococci, or an enterococcal cell wall preparation (Drake and Pang, 1989). Bancsi and colleagues investigated the modulation of tissue factor activity (TFA) by *S. sanguis* and monocytes both *in vitro* and *in vivo*. An *in vitro* model of an endocardial vegetation revealed that although adherence of monocytes to fibrin alone was sufficient to induce TF expression, the presence of *S. sanguis* significantly increased this expression (Bancsi *et al.* 1994). Endothelial cells, fibroblasts and monocytes can all express surface TF and thus may be responsible for TFA in endocardial vegetations. To determine whether monocytes were responsible for this activity, rabbits were treated with etoposide (a cytostatic drug) to induce selective monocytopaenia before inducing *S. sanguis* experimental endocarditis (Bancsi *et al.* 1996). TFA in vegetations of monocytopaenic rabbits was significantly reduced compared to untreated controls, although the weight of vegetations was the same. In rabbits treated with penicillin, the reduction in the number of colonising bacteria correlated with a reduction in TFA in the vegetations. This was suggested to indicate that the presence of bacteria upregulated TF expression by monocytes. However, the numbers of monocytes present in the vegetations was not determined, and so decreased TFA may have simply reflected an overall decrease in monocytes in the vegetation (and this might feasibly have occurred as a result of lower bacterial numbers releasing less bacterial-derived leukocyte chemoattractant).

S. sanguis (strain FSS2) has been reported to express a thrombin-like activity and a Hageman factor-like activity, both of which are coagulation factors in humans (Mayo *et al.* 1995). This activity was reported to be modulated by growth conditions, (activity was five-fold higher at pH 7.5 than pH 5.5), and was thus suggested to be consistent with an activity that has greater importance in tissues than on tooth surfaces.

1.2.4.6 Tissue-degrading enzymes

Enzymes produced by streptococci have been suggested to be involved in destruction of cardiac valve tissue in infective endocarditis (Straus, 1982; Straus and Portnoy-Duran, 1983). Viridans streptococci produce proteases including elastase (Harrington and Russell, 1994) and collagenase (Willcox *et al.* 1995). *S. oralis* has been reported to express a 146kDa trypsin-like protease on its surface, and a related protease was demonstrated in *S. sanguis* by a polyclonal antiserum raised to the *S. oralis* protease (Lo and Hughes, 1996).

1.3 Inflammation

Infective endocarditis, by definition, involves inflammation of the affected tissues. When tissues are damaged or infected, changes occur in the microcirculation that lead to the four cardinal signs of inflammation: redness ("rubor") and warmth ("calor"; due to vasodilation), swelling ("tumour"; due to vasodilation and the accumulation of a cellular and fluid exudate), and often pain ("dolor"; due to tissue swelling and the presence of pain mediators). Circulating leukocytes move from the bloodstream out into the tissues by diapedesis (active movement between the endothelial cells of the blood vessels). Polymorphonuclear leukocytes are the predominant cell to infiltrate the tissues initially, but die within a couple of days. Mononuclear cells, particularly macrophages, become more evident in the later stages once the acute inflammation subsides. The changes seen in the initial stages of the inflammatory response are caused by the release of preformed inflammatory mediators, including histamine released by mast cells, kinins derived from precursor proteins found in plasma and tissues, and the complement activation products C3a and C5a (which have both

chemotactic and histamine-releasing properties). In bacterial infections the microorganisms themselves release inflammatory materials such as formyl-Met-Leu-Phe (fMLP), a molecule with potent chemotactic activity. During the later stages of inflammation, prostaglandins and leukotrienes are produced from leukocytes, endothelial cells and platelets, and both molecules mediate and control the inflammatory response (Roitt, 1997).

If inflammation becomes more severe the body mounts a general metabolic response called the *acute phase response.* This is characterised by increased secretion of corticosteroid hormones (which have a general dampening effect on inflammation), fever, changes in serum metal ion content and acute phase protein synthesis by the liver. The acute phase proteins include protease-inhibitors, fibrinogen, serum amyloid protein, C-reactive protein and ceruloplasmin (an antioxidant). Many of the features of the acute phase reaction are due to the action of interleukin-1, a cytokine that is released by activated macrophages during inflammation (Henderson, 1998).

If the initial response fails to remove the bacteria, the result will be chronic inflammation, a situation that results from a constant leakage of microbial products and antigens from the site of infection, and continued attempts by immune cells to eliminate the causal agents. In contrast to infective endocarditis caused by *Staph. aureus* (which tends to be an acute, quickly progressing, disease), streptococcal endocarditis usually takes a slower course, resulting in chronic inflammation. The aggressive nature of *Staph. aureus* endocarditis is reflected in the high case-fatality rate, which may be greater than 40% (Roder *et al.* 1999), while endocarditis caused by viridans streptococci generally has a cure rate of 90% or more (Gold, 1992).

Although inflammatory reactions are an important part of host defences in eradicating infectious agents, they can also be damaging to host tissues. Particularly in chronic inflammation, the persistent nature of the reaction results in slow tissue destruction despite attempts at repair by fibroblasts. This is likely to have serious consequences for the host if it occurs in a major organ such as the heart. Repair processes are closely

linked to inflammation, and can also have a detrimental effect on tissues - for example, damaged heart muscle tissue cannot be replaced, but is repaired by the laying down of connective tissue, and as a result the functional properties of the tissue are compromised.

The following sections describe the roles of immune cells and cytokines in inflammatory pathology.

1.3.1 Immune cell involvement in infective endocarditis

One of the key features of bacterial endocarditis is the protection afforded to the infecting organisms from host immune cells by growing within the vegetation itself. Histological staining of endocardial vegetations reveals that there are few immune cells present in the platelet fibrin matrix, but that mononuclear cells may be associated with the surface of the vegetation (Curran, 1985). Staining also shows that the underlying tissue is subject to infiltration by large numbers of leukocytes. In so-called "sub-acute" infective endocarditis (as opposed to the more aggressive "acute" form of the disease that tends to be due to *Staph. aureus*) the inflammatory cells present in the tissues reflect the chronic nature of the infection: mononuclear cells, lymphocytes and plasma cells, but few neutrophils, are present (McFarland, 1992). Neutrophils predominate in "acute" infective endocarditis. Experiments in animal models have suggested that the latter contribute to valve damage in infective endocarditis (Freedman, 1987).

1.3.2 Immune cells and tissue pathology

One of the primary defense strategies of the immune response is the killing of microbes by oxygen-dependent mechanisms. This is carried out by macrophages and neutrophils, and is achieved by the production of reactive oxygen species which are converted to hydrogen peroxide by the enzyme superoxide dismutase. In addition, neutrophils possess myeloperoxidase which mediates the formation of hyperchlorous acid (HOCl) from hydrogen peroxide and chloride ions; this is known as the "halogenating system" and provides phagocytes with very potent microbicidal activity. Superoxide anions,

hydrogen peroxide, hydroxyl radicals and halogenated compounds are all potent microbicidal molecules, with hydrogen peroxide and halogenated compounds being better able to exert effects on microbes in the extracellular vicinity since they are more stable molecules and can thus diffuse further. Reduced oxygen species that escape from macrophages or neutrophils may damage host tissue cells and connective tissue matrix (Weiss, 1989), in particular HOCl, which has been implicated in phagocyte-associated inflammation and tissue injury (Weiss, 1986).

During phagocytosis (whether by neutrophils or macrophages), lysosomal granule contents are released into the phagocytic vacuole to kill the engulfed bacteria. Neutrophils have two types of granules: (i) azurophil granules which contain myeloperoxidase, lysozyme, elastase, cathepsin G (a neutral proteinase), defensins (cationic antimicrobial peptides), acid hydrolases and bactericidal permeability-increasing protein (BPI); and (ii) specific granules which contain lysozyme, cytochrome b558 (a plasma membrane enzyme responsible for the reduction of molecular oxygen to superoxide anion), OH-phosphatase, and lactoferrin. Cells of the monocyte/macrophage lineage possess a similar armoury of enzymes including myeloperoxidase, elastase, collagenase and lysozyme. During phagocytosis, lysosomal granule contents may escape into the surrounding tissues before the phagocytic vacuole is completely sealed off (Weissmann *et al.* 1980). This leakage is increased when phagocytes encounter difficulty in engulfing particles (termed "frustrated phagocytosis"), perhaps due to the large size of the target. The escape of lysosomal granules results in acid hydrolases and proteases, particularly elastase, coming into close contact with substrates in the surrounding tissues that they can cleave and damage. Both neutrophil and macrophage enzymes, such as elastase and other matrix metalloproteinases, are purported to play an important role in the tissue pathology of pulmonary emphysema (Kanazawa *et al.* 1992; Finlay *et al.* 1997).

1.3.3 The cytokines

Cytokines are low molecular weight proteins that mediate their effects via high affinity cell-surface receptors on target cells. In contrast to hormones, the cytokines tend to act

in a paracrine manner, exerting their effects over relatively small distances (although, for example, IL-1, IL-6 and TNF can have systemic effects such as the induction of fever). They may also act in an autocrine manner, for example IL-1β produced by monocytes can induce IL-1β release from the same cell type (Dinarello *et al.* 1987). Interest in the cytokines began with investigations into infection and immunity - the first cytokine discovered was the pro-inflammatory cytokine IL-1, termed "endogenous pyrogen" because of its fever-inducing activity - although it is now widely accepted that the cytokines are key signalling molecules in the homeostasis of cellular and tissue activites, and even in development. Over 100 cytokine gene products have been identified, and their effects are wide-ranging, both in terms of the cells they target, and the biological consequences. Many have overlapping biological activities (or "redundancy") - for example IL-1, TNFα, IL-6 and IFNγ are all capable of inducing fever (Dinarello, 1989). Due to their high level of pleiotropy, the cytokines are difficult to group: on whatever basis they are classified, many fall into multiple categories. The cytokines can be roughly divided into six families based on their biological actions.

1. The interleukins (e.g. IL-1, IL-2 etc.) were named after the fact that they were produced by leukocytes and acted to modulate the activities of other leukocytes. However, interleukins are now accepted to be produced by a variety of cell types other than leukocytes.

2. Cytotoxic cytokines (e.g. TNFα) are involved in cell killing or inhibition of cell growth, but are also potent pro-inflammatory molecules.

3. The interferons (e.g. IFNγ) play a major role in inhibiting virus growth and spread, and in the control of leukocyte function.

4. The colony-stimulating factors (e.g. G-CSF) are involved in the growth and differentiation of monocytes and polymorphonuclear cells, and also play a role in

inflammation through interactions with mature cells. IL-1 shares some of its activities with members of this group.

5. Growth factors, as their name suggests, stimulate the proliferation of their target cells, although some have additional activities and the TGFβs are actually inhibitors of cell proliferation.

6. Chemokines are chemotactic peptides that can be divided into four subgroups based on the arrangement of the first two of four conserved cysteine residues that are linked by disulphide bonds. The C-X-C chemokines (e.g. IL-8) tend to attract neutrophils, while the C-C chemokines (e.g. MIP-1α and RANTES) generally attract monocytes. The C chemotaxin, lymphotactin, which attracts T-lymphocytes, is counted as a third class of chemokine (Kelner *et al.* 1994), and the fourth class is the C-X_3-C chemokines (fractalkine/neurotactin) (Bazan *et al.* 1997).

1.3.3.1 Evidence for cytokine involvement in inflammatory tissue pathology

A finely-tuned balance exists between pro- and anti-inflammatory cytokines in healthy tissues. It is now well established that infectious inflammatory disease involves interference with these cytokine networks. In certain diseases the resultant pathology is due to an inappropriate and damaging cytokine response. For example, septic shock results from the overproduction of IL-1 and TNFα (Glauser *et al.* 1994). TNFα has also been implicated in multiple sclerosis and inflammatory bowel disease (Kollias *et al.* 1999). Of the chronic inflammatory diseases, rheumatoid arthritis is probably the best understood example in which IL-1 and TNFα play a damaging role.

1.3.3.1.1 Cytokines in the pathology of rheumatoid arthritis

Rheumatoid arthritis is the prototypic chronic inflammatory disease, involving the articulatory joints of the body. The disease affects the synovial joints causing inflammation of the synovial lining, and this is associated with destruction of synovial

cartilage and subchondral bone. Overproduction of connective tissue matrix components (fibrosis) is also seen in association with the chronic inflammation. Many cytokines have been found in rheumatoid joints (Henderson *et al.* 1995), the most recent addition to the list being IL-17 (Chabaud *et al.* 1999), and there is much evidence that points to a pivotal role for IL-1 and TNF in the pathology of this disease. In *ex vivo* experiments the addition of IL-1 or TNF to articular cartilage explants or foetal bone induces resorption of the extracellular matrices of these tissues (Saklatvala, 1989). *In vivo* experiments showed similar results: local injection of IL-1 into rabbit knee joints stimulated cartilage matrix destruction (Pettipher *et al.* 1986), and systemic administration of IL-1 induced bone breakdown in rodents (Boyce *et al.* 1989). IL-1 thus appears to be an important cytokine in terms of the tissue damage characteristic of rheumatoid arthritis. The synthesis of IL-1 in this disease has been demonstrated to be dependent on the activity of TNFα (Brennan *et al.* 1989), and since the discovery of a linear cytokine cascade with TNFα at the top, various attempts have been made to turn this to therapeutic use by the administration of agents that block TNFα. Several strategies aimed at targetting TNF have been tried, including cA2, a neutralising monoclonal antibody to TNFα (Lorenz *et al.* 2000), Etanercept, which is a soluble TNF receptor (Garrison and McDonnell, 1999), and even vaccination to induce an antibody response against TNFα (Dalum *et al.* 1999). However, TNF-independent IL-1 production is evident in the murine arthritis model, and it has thus been suggested that both cytokines may need to be blocked to ensure not only a reduction in inflammation (thought to be TNFα-mediated), but also a reduction in cartilage and bone destruction (thought to be mediated by IL-1) (Joosten *et al.* 1999; van den Berg *et al.* 1999).

1.3.3.2 Evidence for the involvement of cytokines in infective endocarditis

In any disease involving inflammation, cytokines play a central role. As far as infective endocarditis is concerned, there are a number of reports suggesting cytokine involvement. In one study, circulating levels of IL-6 and TNFα, measured in patients with enterococcal or streptococcal endocarditis, were found to be low or undetectable by commercial immunoradiometric assays with detection limits of 5 to 10pg/ml (Kern

et al. 1993). Levels of soluble TNF receptors were significantly elevated in endocarditis patients compared with those of healthy controls. However, at least with IL-6, there are problems associated with the measurement of circulating cytokines, in that most is present in a "chaperoned" state, in complexes with binding proteins including soluble IL-6 receptors (sIL-6R). These complexes exhibit varied reactivity in different immunoassays and may result in underestimates of circulating IL-6 levels (Sehgal, 1996). *In vitro* assays demonstrated that monocytes from patients with Q-Fever endocarditis (caused by *Coxiella burnetii*) spontaneously (i.e. in the absence of an exogenous stimulus such as LPS) secreted significantly more IL-1β and TNFα (Capo *et al.* 1996b), and IL-10 (Capo *et al.* 1996a) than cells from healthy controls. Transcripts for IL-1β, TNFα and IL-6 were also upregulated in cells from infected patients compared to controls (Capo *et al.* 1996b). The same group also demonstrated that circulating levels of TNFα, IL-6 and soluble TNFα receptor (TNF-R) were significantly higher in patients with Q-fever endocarditis than controls (Capo *et al.* 1999). Later work revealed that TNF-R75, but not the more widely expressed TNF-R55, was specifically upregulated in Q-fever (Capo *et al.* 1999; Ghigo *et al.* 2000).

More direct evidence that cytokines are involved in the inflammation associated with infective endocarditis comes from work by Standiford and co-workers. Immunohistochemical staining of human endocardial tissue from patients with *Staph. aureus* endocarditis revealed that cell-associated IL-8 was expressed by mononuclear phagocytes (Standiford *et al.* 1994). The same group later demonstrated the expression of cell-associated MIP-1α by macrophages, neutrophils and fibroblasts in inflamed areas of endocardium (also from cases of *Staph. aureus* endocarditis) (Danforth *et al.* 1995).

1.3.4 Streptococci induce cytokine secretion

A number of reports describe the ability of whole streptococci or their components to induce cytokine secretion in human cells. However, results from different groups can be difficult to compare due to the use of different cell types, and the use of biological

assays for cytokines, which may have different sensitivities to enzyme-linked immunosorbent assays (ELISA). Biological assays also do not take into account the potential for co-induction of cytokine receptors which may lead to underestimations of cytokine production. In addition, the issue of contamination of supposedly pure preparations with the potent cytokine-inducing molecule LPS is not always fully addressed.

1.3.4.1 Whole bacteria

There are numerous reports of whole streptococci inducing cytokine synthesis in various experimental systems. Whole *S. sanguis* was demonstrated to induce higher levels IL-1 and TNF from human PBMC than *Porphyromonas gingivalis* and *A. actinomycetemcomitans* (Kjeldsen *et al.* 1995). Vernier and colleagues demonstrated the ability of various species of oral streptococci to induce KB cells (a human epithelial cell line) to secrete IL-8, and human saphenous vein endothelial cells to secrete both IL-8 and IL-6 (Vernier *et al.* 1996). Intact heat-killed *S. pneumoniae* induced TNFα and IL-10 release from human monocytes (van Furth *et al.* 1995), and IL-1β, IL-6 and TNF from both whole blood and the human monocytic cell line, THP-1 (Cauwels *et al.* 1997). More recently, the spectrum of cytokines induced by heat-killed *S. pneumoniae* in PBMC has been extended to include IL-8, IL-12, TNFβ, and IFNγ (Arva and Andersson, 1999). Muller-Alouf and colleagues demonstrated that whole heat-killed *S. pyogenes* stimulated human monocytes to secrete IL-1, IL-6 and TNFα (Muller-Alouf *et al.* 1994), and live *S. pyogenes* induced human PBMC to express IL-1β, IL-6, IL-12, IL-18, TNFα and IFNγ, measured in terms of both mRNA and protein (Miettinen *et al.* 1998). Intact *S. mutans* was demonstrated to stimulate human mononuclear cells to secrete IL-12, IFNγ and TNFα *in vitro* (Jiang *et al.* 1999). The binding of whole *S. bovis* to a human buccal epithelial cell line (KB cells), human saphenous vein endothelial cells or THP-1 cells, induced the release of IL-8 (Ellmerich *et al.* 2000). Kwak and colleagues demonstrated the ability of heat-killed Group B streptococci to

induce the secretion of TNFα, IL-1β, IL-6 and IL-8 from human monocytes, as well as IFNγ and IL-12 from lymphocytes (Kwak *et al.* 2000).

1.3.4.2 Lipoteichoic acid

Lipoteichoic acid is a major component of the Gram-positive cell wall, and is thought to be released into the extracellular millieu, particularly in the presence of β-lactam antibiotics (Horne and Tomasz, 1979; van Furth *et al.* 1999). Bhadki and colleagues extracted LTAs from a variety of species of streptococci and tested their ability to induce cytokine secretion from cultured human monocytes (Bhakdi *et al.* 1991). Of the species investigated, only LTA from *S. pyogenes* induced cytokine secretion; LTAs from *S. pneumoniae* and *S. mutans* were inactive, even at 5μg/ml. In another study, by Keller and colleagues, commercial LTA preparations from various species, including *S. mutans* and *S. pyogenes*, as well as LTAs extracted from *S. sanguis* and *S. pneumoniae*, were tested on bone marrow-derived mononuclear phagocytes (Keller *et al.* 1992). Production of TNFα was measured as the cytolytic activity of culture supernatants on TNFα-sensitive cells. All four streptococcal LTAs induced TNFα release, although upwards of 1μg/ml was required for the activity of *S. sanguis* and *S. pneumonia* LTA, with that of *S. pyogenes* and *S. mutans* being approximately 10-fold less potent. In contrast, Standiford and co-workers demonstrated that LTA from *S. pyogenes* was active at as little as 10ng/ml, although the activity measured in these studies was the ability to induce IL-8 release (Standiford *et al.* 1994), and increase the level of MIP-1α mRNA (Danforth *et al.* 1995) in human monocytes. In a later study, LTA from Group B streptococci was demonstrated to induce IL-1β, IL-6 and TNFα release in whole blood assays (von Hunolstein *et al.* 1997).

1.3.4.3 Peptidoglycan

Peptidoglycan is another major component of streptococcal cell walls that has been reported to induce cytokine secretion from human cells. Heumann and colleagues showed that *S. pneumoniae* peptidoglycan stimulated human monocytes to secrete TNFα, as measured by a cell cytotoxicity assay (Heumann *et al.* 1994). A relatively

high concentration of peptidoglycan was required to induce TNFα (greater than 1μg/ml), but the addition of polymyxin B had no effect on this activity.

1.3.4.4 Capsular polysaccharides

The serotype f polysaccharide of *S. mutans* was reported to induce cytokine secretion from human monocytes, and the cytokines produced (IL-1β and TNFα) were demonstrated to be biologically active: TNFα in a cytotoxicity assay, and IL-1β in a thymocyte proliferation assay (Benabdelmoumene *et al.* 1991). Both the type- and group-specific polysaccharides of Group B streptococci were reported to induce the secretion of IL-1β, IL-6 and TNFα in whole blood assays (von Hunolstein *et al.* 1997).

1.3.4.5 Exotoxins

The *S. pyogenes* pyrogenic exotoxin A was demonstrated to induce the release of IL-1α, IL-1β, IL-6, IL-8 and both TNFα and TNFβ from human PBMC, with TNF induction showing an apparent requirement for T cells (Muller-Alouf *et al.* 1994). Houldsworth and colleagues reported that as little as 3pg/ml of *S. pneumoniae* pneumolysin could induce significant IL-1β and TNFα secretion from human monocytes; 10pg/ml induced 50% of the maximal response (Houldsworth *et al.* 1994). This puts pneumolysin on the same level as LPS in terms of potency, and makes it the most potent cytokine-inducing streptococcal molecule described to date.

1.3.4.6 Other components

Soell and colleagues demonstrated that the antigen I/II (a family of adhesins expressed by most oral streptococci)-related SR protein of *S. mutans* induced TNFα release from human monocytes. This was mediated by binding to the carbohydrate portions of monocyte membrane glycoproteins, in particular N-acetylneuraminic acid and fucose (Soell *et al.* 1994). The binding of recombinant antigen I/II polypeptides to the THP-1 cell-line, and subsequent cytokine induction, has also been investigated (Chatenay-Rivauday *et al.* 1998). *S. mitis* culture supernatants were found to contain a protein of greater than 150kDa that stimulated the production of IL-6, TNFα, IFNγ and thymocyte-

activating factor (TAF; presumably IL-1) from human peripheral blood leukocytes (Takada *et al.* 1993). Several other studies have reported cytokine-inducing activity in streptococcal culture supernatants (Bayston *et al.* 1992; Soto *et al.* 1996; Soto *et al.* 1998), although they did not attempt to identify the nature of the active component.

1.3.5 The role of the CD14 antigen in bacterial induction of cytokines

CD14 is a 55kDa glycoprotein that is expressed by myeloid cells and functions as a receptor for ligands from microbial pathogens, such as LPS. It is found as two distinct forms; a membrane-bound form, attached via a glycosylphosphatidylinositol (GPI) anchor, and a soluble serum protein which lacks the GPI anchor (Bazil et al 1986). GPI-linked CD14 is involved in the activation of, and cytokine induction in, myeloid cells (Pugin et al 1994), and the soluble form can facilitate the activation of non-myeloid cells, such as endothelial and epithelial cells (Pugin et al 1993). Membrane-bound CD14 is also involved in ligand internalisation (Poussin et al 1998). Both the soluble and membrane forms bind LPS, and a number of other bacterial molecules have been demonstrated to exert their effects via CD14, including peptidoglycan (PG) (Weidemann *et al.* 1994; Gupta *et al.* 1996; Weidemann *et al.* 1997; Dziarski *et al.* 1998), LTA (Cleveland *et al.* 1996; Hattor *et al.* 1997), lipoarabinomannan (Means *et al.* 1999), and rhamnose glucose polymers found on the cell walls of oral streptococci (Soell *et al.* 1995). CD14 has therefore been suggested to be a pattern-recognition receptor, allowing cells to recognise components from a wide variety of microorganisms (Pugin *et al.* 1994). CD14 lacks a transmembrane domain, and LPS transmembrane signal transduction is dependent on the formation of a complex with the Toll-like receptor 4 (TLR4) and MD2 (Shimazu *et al.* 1999). In contrast, other CD14-dependent ligands, such as LTA, lipoarabinomannan and PG, have been shown to require TLR2 in complex with CD14 to mediate their effects (Schwandner *et al.* 1999).

1.4 Hypothesis

Most research into the pathogenesis of infective endocarditis to date has focused on the interactions between bacteria and platelets and the development of the characteristic thrombotic vegetation. The subsequent inflammation and tissue damage that occurs has not been studied to any great extent and may provide vital clues to alternative treatments.

Any inflammation to the heart's tissues, however small, is likely to pose serious problems to the host. The enigma is that the bacteria most frequently isolated from endocardial vegetations (oral streptococci) are commensals, living on or in the host without causing any detrimental effects. Yet when the same organisms find themselves in a different environment, such as the heart, they are capable of causing a life-threatening disease. An explanation for this may lie in the cytokine networks of the host's immune system.

In the healthy host there is a balance between pro- and anti-inflammatory cytokines, but if this balance is upset (by bacterial components or other stimuli) the result is inflammation and tissue damage. The pathology of infectious inflammatory diseases is known to involve the bacteria's ability to interfere with host cytokine networks. Many components of bacteria have been reported to modulate the balance of cytokine production and have thus been termed "modulins" (Henderson *et al.* 1996a). The majority of these molecules are structural components of microbes. With the oral streptococci in particular, little research has been carried out into the role of secreted or "exported" molecules as modulins. In previous work at the Eastman Dental Institute, transmission electron microscopy of several bacterial species stained with ruthenium red revealed an electron dense layer surrounding the cells which could be removed by gentle washing with saline. The resulting extracts contained a large number of proteins and exhibited various biological activities. Potent cytokine-inducing properties were demonstrated in the material extracted from *A. actinomycetemcomitans*, (the material was significantly more potent than LPS extracted from the same bacterium) and this

was suggested to have possible relevance in the pathophysiology of periodontal disease (Reddi *et al.* 1996b). A 2 kDa peptide was found to be responsible for this activity (Reddi *et al.* 1996a). Turning to the Gram-positive organisms, both *Staph. aureus*, and *Staph. epidermidis* have been shown to produce proteinaceous extracellular material with cytokine-modulating properties (Crean *et al.* 1996; Crean *et al.* 1997; Crean *et al.* 1998).

The hypothesis proposed in this thesis is that the exported proteins of *S. sanguis*, are capable of tipping the balance of cytokines in favour of inflammation, causing endocardial tissue damage via the host's own immune response. The identification of modulins from *S. sanguis* may provide new targets for the development of therapeutic agents.

1.4.1 Aims

1.To extract and characterise the exported material from *S. sanguis* cultured in the presence or absence of blood.

2.To determine the cytokine-inducing (or inhibiting) activity of the exported proteins.

3.To isolate the components responsible for the cytokine-modulating activities.

CHAPTER 2

MATERIALS AND METHODS

This section describes the general materials and methods used throughout this study. Methods particular to individual chapters are detailed within the relevant chapter. Unless otherwise stated, all reagents were from BDH, UK.

2.1 Bacterial strains and routine culture

S. sanguis NCTC 10904, a strain originally isolated from plaque, was used in this study. Bacterial stocks were stored at -70°C in nutrient broth (Oxoid, UK) containing 10% glycerol. Cultures were routinely grown on Wilkins Chalgren agar (WCA) (Oxoid) at 37°C under anaerobic conditions and subcultured every two days. Purity of cultures was checked by Gram-stain.

2.2 Protein Assays

2.2.1 Bio-Rad DC assay

The protein concentrations were determined by the Bio-Rad DC (detergent compatible) protein assay kit (Bio-Rad, UK) in accordance with the manufacturers instructions. The Bio-Rad assay is based on the same chemistry as the Lowry assay, primarily detecting tyrosine and tryptophan residues. Bovine serum albumin (BSA, Sigma, UK) standards ranging from 10μg/ml to 2mg/ml were prepared in PBS. A blank consisting of PBS was also included. Samples were diluted in PBS. 20μl of sample or standard was pipetted in triplicate into a 96 well microtitre plate. 10μl of reagent A was added to each of these wells, followed by 80μl of reagent B. The plate was mixed by gentle shaking and incubated at 37°C for 15 minutes. Using a Dynex plate-reader and software (Dynex, USA), the absorbance at 650nm was read, and a standard curve produced from the BSA standards. The concentrations of protein in the unknown samples were calculated from the standard curves using the Dynex software.

2.2.2 Lowry assay

This assay was carried out according to the method of Lowry *et al* (Lowry *et al.* 1951). A 1mg/ml stock solution of BSA was diluted in PBS to give the following concentrations as standards: 1.6, 8, 40, 200 and 1000μg/ml. EM was also diluted in PBS to give the following concentrations: 0.12, 0.6 and 3mg/ml. 500μl of standard or sample was added to a 10ml glass test tube. 2.5ml of Reagent A (0.1M NaOH, containing 2% (w/v) Na_2CO_3, 0.02% (w/v) sodium potassium tartrate, and 0.01% (w/v) $CuSO_4$) was added to each tube and vortexed immediately, and then left to stand at room temperature for 10 minutes. 250μl Folin-Ciocalteau's reagent (BDH), diluted 1 in 2 with distilled water, was added to each tube, vortexed immediately and left to stand for 30 minutes. 1ml from each tube was transferred to a plastic cuvette and the absorbance at 750nm measured using a Pharmacia spectrophotometer (AmershamPharmaciaBiotech, UK).

2.2.3 Bradford (dye-binding) assay

This was carried out following the method of Bradford (Bradford, 1976). The following dye solution was prepared in a 1 litre volumetric flask: 0.01% Coomassie Brilliant Blue G-250 (Sigma), dissolved in 8.5% phosphoric acid and 4.75% ethanol. The solution was filtered through Whatman No.1 filter paper (Millipore, UK) before use.

A 1mg/ml stock solution of BSA was diluted in 0.15M NaCl to give the following concentrations as standards: 25, 50, 75 and 100μg/ml. Freeze-dried EM was also diluted in 0.15M NaCl to give the following concentrations: 0.12, 0.6 and 3mg/ml. 100μl of standard or sample was added to a 5ml test tube, and 1ml of dye solution added to each. Tubes were vortexed and allowed to stand at room temperature for 2 minutes before transferring 1ml to a plastic cuvette. Absorbance at 595nm was measured using a Pharmacia spectrophotometer.

2.2.4 Lipopolysaccharide assay

The amount of lipopolysaccharide (LPS) (often used interchangeably with the term "endotoxin") contamination present in the samples was measured by the *Limulus* amoebocyte lysate (LAL) assay. All reagents were obtained from Associates of Cape Cod Inc. (Massachusetts, USA), including pyrogen-free water. 96 well assay plates were obtained from Sarstedt (UK). Only reagents and plasticware certified to be pyrogen-free were used for this assay.

Stock LPS was diluted in pyrogen-free water to give the following concentrations for use as standards: 0.5, 0.25, 0.125, 0.06, 0.03, 0.015, 0.0075 IU/ml. Samples for testing were diluted in pyrogen-free water to give a top concentration of approximately 200μg/ml, and ten-fold dilutions performed to give the lowest concentration of at least 200pg/ml. Where the initial concentration of a sample was unknown, ten-fold dilutions were prepared from the undiluted stock. Negative controls of pyrogen-free water only were included. Lysate was spotted onto the 96 well plate cover, to form a 9μl droplet of liquid in the middle of each rimmed circle. Starting with the most dilute standard or sample, 9μl (in duplicate) was pipetted directly into the droplet of lysate. The plate lid was covered and incubated at 37°C in a non-CO_2 incubator for exactly 1 hour. Using a fine pipette tip or Hamilton syringe, 1μl of 0.2% methylene blue (Sigma) in 70% ethanol was spotted onto each mixed droplet (again starting from the most dilute sample/standard). Negative results (i.e. samples containing no LPS) were shown by the formation of a homogeneous blue droplet, whereas a positive result (i.e. a sample containing LPS) was shown by the exclusion of the dye due to gelling of the lysate, and the formation of a blue star on top of the droplet. The test was recorded as valid if the LPS standards gelled one dilution above or below the lysate label claim as printed on the bottle.

2.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

2.3.1 Reagents

SDS	10% solution (w/v)
Stacking gel buffer	0.5M Tris-HCl pH 6.8
Resolving gel buffer	1.5M Tris-HCl pH 8.8
Electrophoresis buffer (5x)	0.125M Tris, 0.96M glycine, 0.5% SDS, pH 8.3
30% Acrylamide	29.2% (w/v) acrylamide: 0.8% (w/v) bis-acrylamide (37.5:1) (National Diagnostics, UK)
Ammonium persulphate (APS)	10% solution, prepared fresh (Sigma)

10µl of TEMED (N,N,N',N'-tetramethylethylenediamine) (Sigma) and 100µl of APS were added per 10ml to induce polymerisation of the gels. The final composition of the gels was as follows:-

Stacking gel

4.8% acrylamide

0.125M Tris-HCl, pH 6.8

0.1% SDS

Resolving gel

6-12% acrylamide, depending on required separation range

0.375M Tris-HCl, pH 8.8

0.1% SDS

2.3.2 Resolution of proteins by SDS-PAGE

Samples were mixed with reducing sample buffer (Pierce & Warriner (UK) Ltd.) at 4 parts sample to 1 part sample buffer, and heated in a boiling water-bath for 5 minutes. For gels destined for electro-blotting and N-terminal sequencing, the following 4 x

sample buffer was used instead: 0.25M Tris-HCl pH 6.8 containing 8% (w/v) SDS, 50% glycerol and 0.1M dithiothreitol (DTT) (Sigma).

Silver Stain SDS-PAGE Molecular Weight Standards (Sigma) were run on all gels destined for silver staining. For other gels and Western blots, Broad Range protein markers were used (NEB, UK). Proteins were separated by SDS-PAGE using the method of Laemmli (Laemmli, 1970). A Mini PROTEAN II electrophoresis system was used (Bio-Rad). Initially electrophoresis was carried out at 30mA per gel until the samples were through the stacking gel, after which the current was reduced to 15mA per gel for separation through the resolving gel.

2.3.3 Silver staining

The silver staining protocol was adapted from the method of Morrisey (Morrissey, 1981). Gels were fixed in 40% methanol, 10% acetic acid in distilled water for 30 minutes, followed by 30 minutes in 5% methanol, 7% acetic acid in distilled water. Gels were then washed in at least 2 changes of distilled water for a total of 1 hour, and then 30 minutes in 100ml of 5μg/ml DTT. After a 30 minute soak in 100ml of 0.1% silver nitrate (Sigma), gels were rinsed twice with 50ml of developer (3% Na_2CO_3, 0.0185% formaldehyde (Solmedia Laboratory Supplies, UK)), before incubating in the dark with a further 100ml of developer. Development was stopped by the addition of 5ml of 2.3M citric acid.

2.3.4 Colloidal blue staining

After electrophoresis, gels were fixed for 1 hour in 7% acetic acid in 40% methanol. A 1x working solution of Brilliant Blue G-Colloidal Concentrate (Sigma) was prepared by adding 800ml of distilled water to the bottle of concentrate. Four parts of 1x working solution were mixed thoroughly with one part methanol, and the gels incubated in this solution for up to four hours with shaking. Gels were destained with 10% acetic acid in 25% methanol for 60 seconds with shaking, followed by a rinse and further destaining, both with 25% methanol.

2.3.5 Calculation of molecular masses of proteins separated by SDS-PAGE

The distance travelled by each protein standard was measured and plotted against the log of its molecular mass on a scattergraph. Microsoft Excel was used to plot a line of best fit for the standards, and generate an equation to describe the relationship between the distance travelled and the log of the molecular mass. The equation was then used to calculate the molecular mass of unknown proteins from the distance they had travelled in the gel. Only the molecular mass of proteins falling within the range travelled by the standards were calculated. An example of a standard curve with a line of best-fit and equation is shown in Figure 2.1.

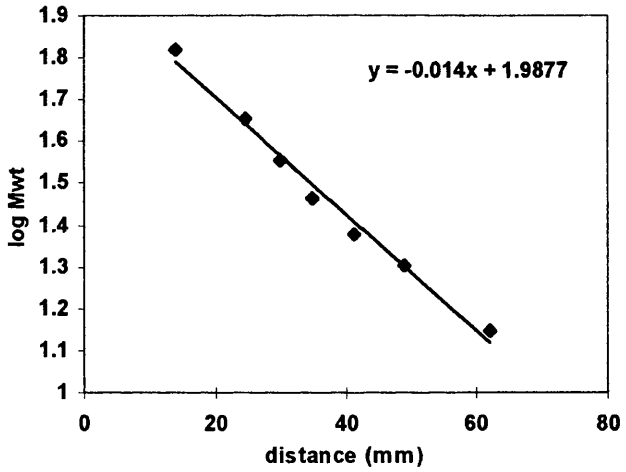

Figure 2.1. The distance travelled by each protein standard was plotted against the log of its molecular mass. A line of best fit was plotted and the equation that describes this line is shown on the graph.

2.4 Electro-blotting of proteins

Two alternative methods were employed, depending on the subsequent use of the proteins.

2.4.1 Electro-blotting using the Tris-Glycine buffer system

This method, using the buffer system described by Towbin and co-workers (Towbin *et al.* 1979), was used for blotting proteins which were to be probed with antisera.

Transfer buffer:-
0.025M Tris
0.2M glycine
20% methanol

After electrophoresis, the gel was soaked in transfer buffer for 30-60 minutes. A nitrocellulose membrane (Millipore) was also soaked in transfer buffer. The gel and membrane were assembled into a "sandwich" with blotting paper and sponges (also pre-soaked in transfer buffer), held in place between two plastic grids. The sandwich was placed in a Mini PROTEAN II Western Blotting tank (Bio-Rad) filled with transfer buffer, and the proteins electrophoretically transferred onto the membrane overnight at constant voltage (15V).

2.4.2 Electro-blotting using the CAPS buffer system

This method was used for proteins that were to be subjected to N-terminal sequencing, and was adapted from the method of Matsudaira (Matsudaira, 1987).

After electrophoresis, the gel was soaked in transfer buffer (10mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) in 10% methanol) for 5 minutes to reduce the amount of tris and glycine. Polyvinylidene difluoride (PVDF) membrane (Immobilon P, Millipore) was pre-wetted in methanol and then soaked in transfer buffer. The gel and PVDF membrane were assembled into a "sandwich" as described in 2.4.1. above, and transferred to the blotting tank filled with transfer buffer. Proteins were electro-blotted onto the PVDF membrane for 30-60 minutes at 200mA (the time was varied depending on the molecular mass of the proteins being blotted, as large proteins take longer to transfer).

2.4.2.1 Coomassie blue staining of proteins electro-blotted onto PVDF membranes

Membranes were removed from the sandwich and stained for 5 minutes with 0.1% coomassie blue in 20% methanol, 0.05% acetic acid. They were then destained in 50% methanol for 20 minutes, rinsed with water and air-dried.

2.4.2.2 N-terminal sequencing

Edman degradation was kindly carried out by Angela Paul at Imperial College using a Perkin Elmer peptide/protein sequencer. This procedure involves the sequential removal of one residue at a time from the amino end of the polypeptide. The terminal amino acid reacts with phenyl isothiocyanate to form a phenylthiocarbomoyl derivative, and under mildly acidic conditions a cyclic derivative of the terminal amino acid is released (a phenylthiohydantoin (PTH) amino acid) and analysed with a PTH analyser.

2.5 Peripheral blood mononuclear cell (PBMC) assays

2.5.1 PBMC preparation

All media were pre-warmed to 37°C. Buffy coat blood (either single donor, or mixed donor "residue" blood, obtained from the National Blood Transfusion Centre, Colindale, London, UK) was diluted in an equal volume of RPMI 1640 (Gibco Life Sciences, UK). This was layered onto 15ml aliquots of Lymphoprep (Nycomed, Norway) in 50ml centrifuge tubes (Sarstedt), and centrifuged at 400 x g for 30 minutes at room temperature in an Eppendorf 5810R centrifuge with an A-4-62 rotor. The white layer of leukocytes that formed at the interface with the Lymphoprep was harvested and transferred to a fresh 50ml tube. Cells were washed twice by resuspending in 50ml of RPMI and re-pelleting by centrifugation for 10 minutes at 400 x g. Washed cell pellets were resuspended in RPMI containing 2% heat-inactivated foetal calf serum (FCS; Pierce & Warriner), 2mM L-glutamine and 100 units/ml of penicillin/streptomycin (Gibco Life Sciences). This medium will hereafter be referred to as "culture medium".

The cell suspension was diluted as necessary and the number of cells per ml counted using a haemocytometer.

2.5.2 Routine PBMC assay

PBMC were prepared as described above in section 2.5.1. Cells were diluted to 2 x 10^6/ml in culture medium and dispensed into 24-well tissue culture plates (Sarstedt) at 1ml/well. Plates were incubated for 1 hour at 37°C in 5% CO_2 in air. Cells were washed once with 1ml/well PBS, and the culture medium replaced. In experiments where the LPS-blocking antibiotic polymyxin B was used, the replacement culture medium contained the appropriate concentration of polymyxin B, and the cells were incubated with this medium for 30 minutes prior to the addition of stimuli. All stimuli, including appropriate controls, were added to the wells in triplicate. To confirm the responsiveness of the cells, control wells were stimulated with 10ng/ml *E. coli* LPS (Difco, UK) in all experiments. Where possible, all stimuli (e.g. *S. sanguis* EM, LPS) were added to the wells in a small volume (usually 10-20µl), but when this was not possible, an equal volume of culture medium was first removed from the appropriate wells before the addition of the stimuli. Plates were incubated for 16 hours (unless otherwise stated) at 37°C in 5% CO_2 in air. After this time, plates were centrifuged at 300 x g for 15 minutes (in an Eppendorf 5810R centrifuge) to pellet the cells. Culture supernatants were assayed for their cytokine content by Enzyme-Linked Immunosorbent Assay (ELISA) and, in addition, samples were stored at -70°C in 96-well microtitre plates for analysis at a later date.

2.5.3 Assessment of cell viability

After samples of cell supernatants had been removed from PBMC assays for use in ELISAs, the cells were resuspended by vigorous pipetting and scraping, before transferring to sterile Eppendorfs. Tissue culture plate wells were examined using a light microscope to check that all of the cells had been removed. 100µl of resuspended cells were incubated with 100µl of trypan blue (Sigma) for 5 minutes. Cells were mixed thoroughly and 10µl transferred to a haemocytometer for counting. More than

100 cells were counted for each sample, and triplicate wells were sampled. A positive control of 10μg/ml of LPS (which was included in the PBMC assays) was also included to confirm that the trypan blue procedure was capable of detecting non-viable cells.

2.6 Enzyme-Linked Immunosorbent Assays (ELISA)

All ELISAs were developed and optimised at NIBSC, UK. The IL-6 ELISA protocol is described by Taktak *et al* (1991), and details of the characterisation of the antibodies for the TNFα ELISA (e.g. specificity) are given in Meager *et al* (1987). The general method for the four ELISAs is given here; details of the concentrations of antibodies used, dilutions of cytokine standards etc. for the individual assays are given in Table 2.1. Maxisorp microtitre plates (Nunc, Life Technologies) were coated with 100μl/well of immuno-affinity purified polyclonal antibodies (NIBSC, UK) diluted in PBS. Plates were incubated overnight at 4°C. The wells were decanted and washed three times with wash buffer (0.01M phosphate/ 0.05M NaCl containing 0.1% (v/v) Tween 20). Cytokine standards (NIBSC) were diluted in wash buffer to give a range of concentrations (see Table 2.1.). Supernatants from PBMC assays were diluted with wash buffer. 100μl of standard or supernatant was added to each well as appropriate, and plates were incubated for 2-4 hours at 37°C, with the exception of the IL-1β assay in which plates were incubated overnight at 4°C as this was found to give optimal results. Wells were decanted and washed three times with wash buffer. Biotinylated affinity-purified polyclonal antibodies (ranging from 0.1 to 0.2mg/ml) (NIBSC) were diluted in wash buffer, and 100μl added to each well. Plates were incubated for 1 hour at 37°C before being decanted and washed three times with wash buffer. 100μl of avidin-horseradish peroxidase (Dako Ltd., UK), diluted 1/4000 in wash buffer, was added to each well, and the plates incubated for 30 minutes at 37°C. After washing the plates three times with wash buffer, the wells were developed by the addition of 100μl/well of 0.2mg/ml OPD (1,2-phenylenediamine dihydrochloride orthophenylene diamine) (Sigma) in 0.1M citric acid-phosphate buffer pH 5.0 plus 0.4μl/ml of 30% H_2O_2 (Sigma). The reaction was terminated by the addition of 150μl of 1M H_2SO_4.

The absorbance at 492nm was read and the concentrations of IL-6 calculated from the standard curves using a Dynex plate-reader and software (Dynex, USA).

Table 2.1. Concentrations of antibodies, cytokine standards, and assay supernatants used in the IL-1β, IL-6, IL-8 and TNFα ELISAs.

	IL-1β	IL-6	IL-8	TNFα
Coating antibody	sheep polyclonal	goat polyclonal	sheep polyclonal	sheep polyclonal
Concentration of coating antibody (μg/ml)	0.5	0.125	0.5	0.5
Concentration range of cytokine standards (ng/ml)	0 - 8	0 - 6	0 - 10	0 - 20
Dilution of assay supernatants	1/5	1/10	1/50	1/10
Biotinylated antibody	sheep polyclonal	goat polyclonal	sheep polyclonal	sheep polyclonal
Dilution of biotinylated antibody	1/1000	1/4000	1/2000	1/2000

2.7 Statistical analysis

All experiments were carried out at least twice, except for the testing of fractions from the protein purifications since as much protein as possible needed to be retained for subsequent purification. For most purposes the Student's t-Test was used (using Microsoft Excel) to determine whether differences in amounts of cytokines detected were significant or not. Where four or more samples required comparison with one other sample (for example a control sample), a Dunette's t-Test was used (using MiniTab). This reduces the probability of a Type II error occurring, a problem when multiple Student's t-Tests are performed. The level of significance was set at 0.05.

2.8 Protein purification

2.8.1 Ammonium sulphate precipitation

Unless otherwise stated, protein solutions (pH 7) were adjusted to a final saturation of 85% ammonium sulphate to precipitate the majority of proteins present. This was carried out at 4°C with constant stirring, with the solid ammonium sulphate added slowly over a period of time. The solutions containing ammonium sulphate were left stirring gently at 4°C overnight. Precipitates were harvested by centrifugation for 30 minutes at 10,000 x g using a Sorvall RC-5B Refrigerated Superspeed Centrifuge (DuPont Instruments) with a GSA rotor, at 4°C. Pellets were dissolved in a small amount of pyrogen-free water (Associates of Cape Cod) and dialysed against distilled water using Spectrapor dialysis tubing with a 3.5 kDa cut-off (Medicell International Ltd, UK).

2.8.2 Ultrafiltration using an Amicon Filtration Cell

A Diaflo ultrafiltration membrane (Millipore, UK), with a molecular weight cut-off of 10kDa (YM10) or 30kDa (YM30), was placed in either a 50ml or a 500ml Amicon filtration cell (Millipore), depending on the volume to be concentrated. The sample was added to the filtration cell, which was set on a magnetic stirrer, and filtration carried out using pressurised nitrogen (2 bar) at 4°C. This procedure was carried out to concentrate protein solutions, and in some cases also to remove salts from the sample, in which case several cell volumes of distilled water were flushed through.

2.8.3 Column chromatography

Given here are details of the columns, their preparation and the buffers used throughout this study. Details of the individual fractionation methods are given in the Materials and Methods sections of the appropriate chapters.

2.8.3.1 Ion exchange chromatography

2.8.3.1.1 Ion exchange column details

Details of all the ion exchange columns used in this study are given below:-

Column	MA7Q (Bio-Rad)
Type of chromatography	Anion exchange
Column volume	2.4ml
Column dimensions	5 x 0.78cm
Protein-binding capacity	4mg
Buffer A	20mM Tris pH 8.0
Buffer B	20mM Tris pH 8.0 + 1M NaCl

Column	MiniQ (Pharmacia)
Type of chromatography	Anion exchange
Column volume	240µl
Column dimensions	3.2 x 30mm
Protein-binding capacity	1-1.5mg
Buffer A	20mM Tris pH 8.0
Buffer B	20mM Tris pH 8.0 + 1M NaCl

Column	MiniS (Pharmacia)
Type of chromatography	Cation exchange
Column volume	240µl
Column dimensions	3.2 x 30mm
Protein-binding capacity	1-1.5mg
Buffer A	20mM MOPS pH 6.0
Buffer B	20mM MOPS pH 6.0 + 1M NaCl

Column	Q20 (Bio-Rad)
Type of chromatography	anion exchange
Column volume	20ml
Column dimensions	15 x 113mm
Protein-binding capacity	200mg
Buffer A	20mM Tris pH 8.5
Buffer B	20mM Tris pH 8.5 + 1M NaCl

Column	S20 (Bio-Rad)
Type of chromatography	cation exchange
Column volume	20ml
Column dimensions	15 x 113mm
Protein-binding capacity	200mg
Buffer A	20mM MOPS pH 6.0
Buffer B	20mM MOPS pH 6.0 + 1M NaCl

2.8.3.1.2 Preparation of buffers

All buffers were prepared using HPLC Hipersolve grade water and NaCl. Before use, all solutions were vacuum-filtered through 0.22μm nitrocellulose membranes (Millipore) and gas-exchanged by passing Helium through the solutions for 20-30 minutes.

2.8.3.1.3 Preparation of ion exchange HPLC columns

All ion exchange chromatography columns were prepared by running through at least five column volumes of water, followed by at least five column volumes of buffer A. In addition, fractionation of proteins was preceded by a blank run (i.e. no sample was applied).

2.8.3.2 Gel filtration chromatography

2.8.3.2.1 Gel filtration column details

Column	Superdex 75 (Pharmacia)
Type of chromatography	gel filtration
Column volume	2.4ml
Column dimensions	3.2 x 300mm
Size exclusion limit	100 kDa
Optimal separation range	3-70,000 kDa
Equilibration buffer	0.05M phosphate buffer + 0.15M NaCl pH 7.0

2.8.3.2.2 Preparation of buffers

Phosphate buffer was prepared as described in the Data for Biochemical Research handbook (Dawson *et al*, 1986). Briefly, stock solutions of 0.2M Na_2HPO_4 and 0.2M NaH_2PO_4 were prepared and combined in the proportions stated for 0.2M phosphate buffer of pH7.0. This was then diluted 1 in 4 to give 0.05M phosphate buffer. Hipersolve NaCl was added to a final concentration of 0.15M. As for the other columns, all buffers and water were vacuum-filtered and gas-exchanged before use (see section 2.8.3.1.2).

2.8.3.2.3 Preparation of gel filtration column

Preparation was carried out at a flow rate of 20µl/min. The 20% ethanol storage solution was flushed out with one column volume of water. The column was then equilibrated with at least five column volumes of phosphate buffer; for convenience, equilibration was usually carried out overnight with a total of eight column volumes of phosphate buffer.

2.8.3.2.4 Void volume determination for the Superdex 75 gel filtration column

A gel filtration matrix consists of beads with pores of various sizes into which proteins of small enough size will enter as they pass down the column. Proteins that are too large to enter the pores will flow straight through the column without entering any of the beads, and elute first. The void volume is the volume of solvent required to elute a protein that is too large to enter the matrix beads. The exclusion limit of the column is the molecular mass above which a protein will be excluded from the beads; in the case of the Superdex 75, this value is 100kDa. Dextran Blue is a blue dye (Cibacron Blue) linked to dextran (a water-soluble glucan polymer) to give a molecule of high molecular mass (approximately 2000kDa). Because this molecule is larger than the exclusion limit of the column, the volume at which it elutes will correspond to the void volume of the column.

After equilibration of the column, 1μg of Dextran Blue (Sigma) was injected onto the column. One column volume of equilibration buffer were run through the column, and elution of the Dextran Blue was monitored by absorbance at 280nm.

CHAPTER 3

EXTRACTION AND CHARACTERISATION OF THE EXPORTED MATERIAL (EM) OF *Streptococcus sanguis*

3.1 Introduction

S. sanguis is one of the most common causal agents of infective endocarditis, an inflammatory disease. Aside from the ability to cause platelet aggregation, *S. sanguis* has few known virulence factors to account for the inflammatory pathology of this disease. Soluble proteins exported from the bacterium may play a role in the pathogenesis of infective endocarditis.

In addition to several Gram-negative organisms, both *Staph. aureus* and *Staph. epidermidis* have been shown to produce loosely-adherent extracellular material which can be extracted by gentle stirring with saline (Nair *et al.* 1995; Meghji *et al.* 1997). This extract, which we have termed in this study Exported Material (EM), exhibits a variety of biological activities which are postulated to play a role in pathogenesis. These activities include an ability to modulate the production of cytokines from human cells (Crean *et al.* 1997).

The aim of this section of the study was therefore to isolate and characterise the exported material from *S. sanguis*.

3.2 Materials and methods

3.2.1 Growth of bacteria for the isolation of EM

Bacteria were grown on Wilkin's Chalgren agar supplemented with 5% defibrinated horse blood (Oxoid, UK) at 37°C under anaerobic conditions for 48 hours. Bacteria were harvested by gently scraping with a glass spreader, and collected in sterile 0.15M NaCl at 4°C. Bacteria were centrifuged at 8000 x g in a Sorvall RC-5B Refrigerated Superspeed Centrifuge (DuPont Instruments) using a GSA rotor, for 30 minutes, and the supernatant discarded. The pellets were freeze-dried and the final dry weight recorded.

3.2.2 Extraction of EM

EM was extracted by a modification of the method of Wilson *et al.* (Wilson *et al.* 1985). The bacteria were resuspended in 100ml of 0.15M NaCl per gram dry weight and gently stirred for 3 hours at 4°C. After centrifugation, (30 minutes at 8000 x g, 4°C using a GSA rotor in the RC-5B centrifuge), the supernatant, containing extracted EM, was removed and further centrifuged for 30 minutes at 8000 x g. The supernatant was vacuum-filtered through a 0.45μm PVDF membrane (Millipore), and then dialysed against at least 15 changes of distilled water using Spectrapor dialysis tubing with a molecular weight cut-off of 3.5kDa (Medicell International Ltd, UK). The isolated EM was freeze-dried and stored at -70°C.

3.2.3 Electron microscopy

To assess the efficacy of the EM extraction and to determine whether the bacteria remained intact, transmission electron microscopy was used to observe the surface of the bacteria before and after the saline extraction. This work was kindly carried out by Mrs Nicky Mordan, Electron Microscopy Unit, Eastman Dental Institute. The samples were fixed in 3% glutaraldehyde in 0.1M sodium cacodylate buffer for 1 hour at room temperature. Specimens were partially dehydrated in a graded series of concentrations of ethanol (ranging from 70% to 90%) and then infiltrated with LR White before embedding in resin. Sections were examined using a JEOL 100CXII electron microscope.

3.2.4 Composition of *S. sanguis* EM

3.2.4.1 Protein

The protein content of the EM was measured using several different methods: (i) the Bio-Rad DC assay, (ii) the Lowry assay, and (iii) the Bradford dye-binding assay. All assays are described in Chapter 2, sections 2.2.1 to 2.2.3.

3.2.4.2 Carbohydrate

The carbohydrate content was determined by the method of Dubois *et al* (1956). Pyrex test tubes (10ml) were cleaned thoroughly before use by soaking overnight in acid rinse (Decon), rinsing with distilled water and oven-drying. Glucose standards ranging from 20 to 100μg/ml were prepared with distilled water along with several dilutions of EM (0.1, 1 and 2 mg/ml). A blank of distilled water only was included. 1ml of 5% (w/v) phenol was added to 1ml of standard or sample in test tubes and mixed thoroughly by vortexing. 5ml of concentrated sulphuric acid was added and the tubes vortexed immediately before leaving to stand for 10 minutes. The tubes were then incubated in a water bath at 25-30°C for 10-20 minutes to allow the colour to develop if any sugar derivatives were present. 1ml aliquots were dispensed into cuvettes and the absorbance measured at 490nm.

3.2.4.3 Lipid

10mg of EM was dissolved in 5ml of HPLC-grade water. 5ml of chloroform:methanol (2:1) was added and then vortexed for 10 minutes. The tubes were centrifuged at 3000g for 10 min to separate the two layers. The lower chloroform layer was removed into a pre-weighed glass bijou and placed in a fume cupboard with the lid off to allow the chloroform:methanol to evaporate. The amount of lipid present was measured by weighing the bijou.

3.2.4.4 Nucleic acid

EM was dissolved in dH_2O to a concentration of 1mg/ml, and the concentration of nucleic acid determined using a spectrophotometer (Pharmacia, UK) to measure the absorbance at 260nm.

3.2.4.5 Lipopolysaccharide

The level of endotoxin present in the *S. sanguis* EM was measured by the Limulus Amoebocyte Assay (LAL), details of which are given in Chapter 2, section 2.2.4.

3.2.4.6 One-dimensional SDS-PAGE

30µg of *S. sanguis* EM were separated on 12% SDS polyacrylamide gels and silver-stained to reveal the protein banding pattern as described in Chapter 2, sections 2.3.1 to 2.3.3.

3.2.4.7 Two-dimensional SDS-PAGE

To identify the complete population of proteins present, *S. sanguis* EM was separated by two-dimensional SDS-PAGE. This was carried out using a PROTEAN II xi multi-cell system (Bio-Rad) (following the manufacturers method) powered by a PowerPac 1000 unit (Bio-Rad).

3.2.4.7.1 First dimension

Detergent solution

0.3g CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propane sulphonate)
10 % Nonidet P-40

First dimension tube gel monomer solution

5.5g urea (final concentration 9.2M)
1.5ml 30% acrylamide stock (4.5% final acrylamide concentration)
100µl Bio-Lyte 5-7 ampholyte (Bio-Rad)
400µl Bio-Lyte 3-10 ampholyte (Bio-Rad)
3.4ml dH_2O
0.5ml detergent solution
10µl TEMED
20µl 10% APS

Solution A

10% SDS

0.15M DTT

made up in dH_2O

Iso-urea solution

0.1g DTT

0.2g CHAPS

5.4g urea

500µl Bio-Lyte 3-10 ampholyte

50µl 0.5% bromophenol blue (Sigma)

6ml dH_2O

The first dimension monomer solution was gas-exchanged by bubbling helium gas through the solution for 30 minutes before the addition of the TEMED and APS. The solution was drawn into the glass capillary tubes using a syringe and silicone tubing, and allowed to polymerise at room temperature overnight.

500ml of upper buffer (20mM NaOH) and 4 litres of lower buffer (10mM H_3PO_4) were gas-exchanged for 30 minutes before assembling the tube gels in the electrophoresis tank. 540µg of *S. sanguis* EM was dissolved in 80µl of a 1 in 10 dilution of solution A and heated for 5 minutes in a boiling water-bath. After allowing the sample to cool to room temperature, 1µl of iso-urea solution was added per 10µl of sample. Samples were loaded onto the tube gels using a Hamilton syringe and overlayed with upper buffer. The upper reservoir was filled with sufficient upper buffer to cover the tops of the capillary tubes. Gels were run for 2 hours at 200V followed by 2 hours at 500V and then overnight at 800V. Once electrophoresis was complete, the capillary tubes were removed and stored at -20°C.

3.2.4.8 Second dimension

Resolving gel composition (45ml per gel)

0.4M Tris pH 8.8

12.5% acrylamide

The resolving gel solution was gas-exchanged for 30 minutes prior to the addition of TEMED (11µl per gel) and APS (110µl per gel). Gels were overlayed with water-saturated butanol and allowed to polymerise overnight at room temperature.

Stacking gel composition

0.125M Tris-HCl pH 6.8

0.1% SDS

3.9% acrylamide

The stacking gel solution was gas-exchanged for 15 minutes before adding the APS (150µl per 30ml stacking gel) and TEMED (30µl per 30ml of stacking gel). This was poured onto the resolving gels and, with plastic combs in place, allowed to polymerise for 1 hour.

Transfer solution

0.075M Tris-HCl pH 8.8

3% SDS

0.0015% bromophenol blue

Lower and upper running buffer

0.05M Tris

0.4M glycine

0.4% SDS

The tube gels were allowed to thaw at room temperature, extruded from their glass capillary tubes and then laid onto the surface of the stacking gel along with 150µl of

transfer solution. The slab gels were lowered into the electrophoresis tank containing 4 litres of pre-chilled lower running buffer. Pre-chilled upper running buffer was poured into the top reservoir to cover the gels and make contact with the electrode. Twenty microlitres of Silver Stain SDS Molecular Weight Standards (Sigma) were loaded into the small well on each gel. Gels were run at 35mA per gel for 4-6 hours, and then fixed and silver stained as described in Chapter 2, section 2.3.3 (except that volumes were increased proportionally to the size of the gel).

3.2.4.9 Detection of contaminating horse blood proteins in EM by Western blotting

8µg of *S. sanguis* EM was separated on a 12% SDS polyacrylamide gel (Chapter 2, section 2.3). Also run were positive controls of 10µl each of a 1 in 1000 and a 1 in 10,000 dilution of horse serum (prepared from the horse blood used to supplement the bacterial medium), and 10µl of an *E. coli* lysate (Promega, UK) as a negative control. The gel was blotted onto nitrocellulose using the Tris-glycine buffer system as described in Chapter 2, section 2.4.1.

3.2.4.9.1 Immunodetection with anti-whole horse serum

The entire procedure was carried out on a rocking table. The lane containing the molecular mass markers was cut from the rest of the membrane and stained for 1 minute in Amido Black (Sigma) before destaining for 10 minutes in 50% methanol 7% acetic acid. Membranes were washed in PBS + 1% Tween (BDH) for 5 minutes followed by a further 25 minutes in PBS + 0.1% Tween (wash buffer). They were then blocked for 1 hour in wash buffer containing 5% skimmed milk (Sainsbury's, UK), before incubating for 1 hour with rabbit anti-whole horse serum (Sigma) diluted 1 in 1000 in PBS containing 0.1% BSA (Sigma). Six 10 minute washes in wash buffer were carried out, after which the membranes were incubated for 1 hour in goat anti-rabbit HRP conjugate (Dako) diluted 1 in 500 in wash buffer containing 5% skimmed milk. Washes were repeated as described above, and the blots developed using Sigma Fast DAB (3,3'-diaminobenzidine tetrahydrochloride) tablets, prepared according to the

manufacturers instructions. The colour development was stopped by the addition of distilled water.

3.3 Results

3.3.1 Electron microscopy

Before the saline extraction, transmission electron microscopy of ruthenium red-stained *S. sanguis* revealed a substantial layer of extracellular material surrounding the bacteria, including fibrillar structures (figure 3.1a). Saline extraction resulted in the removal of almost all of this layer (figure 3.1b). Cells appeared to remain intact after the extraction, and no cell fragments were observed in the sections examined by electron microscopy.

3.3.2 Composition of EM

The percentage yield in terms of dry weight of EM extracted from a known dry weight of bacteria ranged from 4 to 5%.

3.3.2.1 Protein

The protein content of the EM was measured using three different assays. The Bio-Rad DC assay and the Lowry assay gave results of 24 and 18% respectively, while the Bradford dye-binding assay gave a protein content of just 2.4%. Subsequent batches of EM were assayed for their protein content by the Bio-Rad DC assay and varied from 13% to 33%.

3.3.2.2 Other components

The carbohydrate content of EM grown in the presence of blood, as measured using the Dubois assay with glucose as a standard, ranged from 3 to 4%. The endotoxin content of the EM was <0.00002%. The amount of lipid extracted by the chloroform/methanol method was too small to be accurately weighed. This method requires more than 10mg of EM as starting material, and there was not enough material available to repeat the extraction. Nucleic acid acounted for less than 3% of the dry mass of the EM.

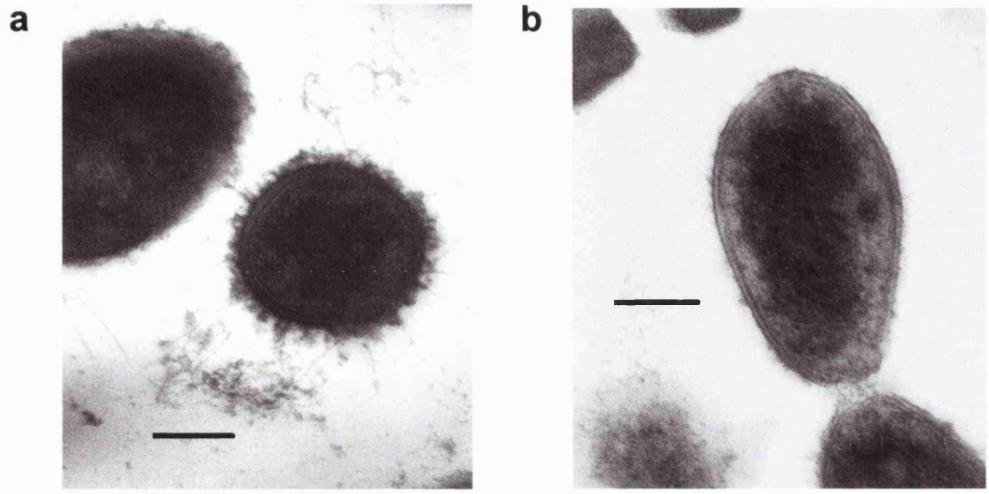

Figure 3.1 Thin sections of *S. sanguis* before (a) and after (b) extraction with saline. The fuzzy outer layer of exported material (EM) is clearly visible in (a), but not in (b). Black bars represent 0.25μm in both (a) and (b).

3.3.3 One-dimensional SDS-PAGE

EM from *S. sanguis* was separated by SDS-PAGE on a 12% gel and silver-stained to disclose the banding pattern of the macromolecules present (figure 3.2). The material contained in excess of 30 macromolecules, presumably polypeptides, with molecular masses ranging from 14 to > 100kDa. Each new batch of EM was subjected to SDS-PAGE and silver-staining, and there was found to be little variation in protein profiles between different batches.

3.3.3.1 Two-dimensional SDS-PAGE

Separation of 540μg of *S. sanguis* EM (equivalent to 180μg protein) by two-dimensional SDS-PAGE revealed a heterogenous mixture of proteins (figure 3.3). Although the separation was rather disappointing, with most of the proteins present in a fairly restricted pH range, approximately 45 to 50 protein spots were visible with silver stain.

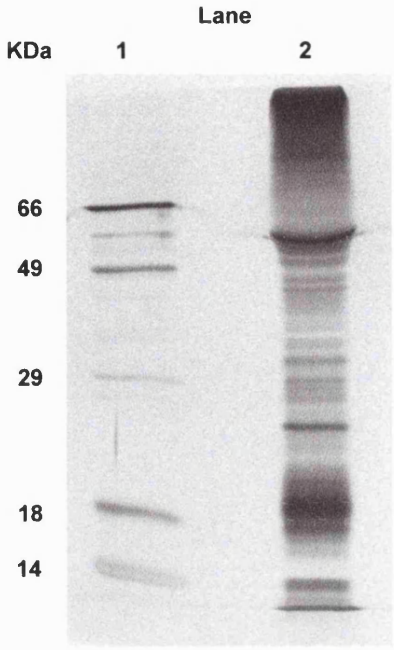

Figure 3.2 SDS-PAGE analysis of EM from *S. sanguis* grown in the presence of blood. 30μg EM (equivalent to 5μg protein) was separated on a 12% SDS polyacrylamide gel and silver stained to disclose the banding pattern. Lane 1 shows molecular mass markers; lane 2 shows the *S. sanguis* EM.

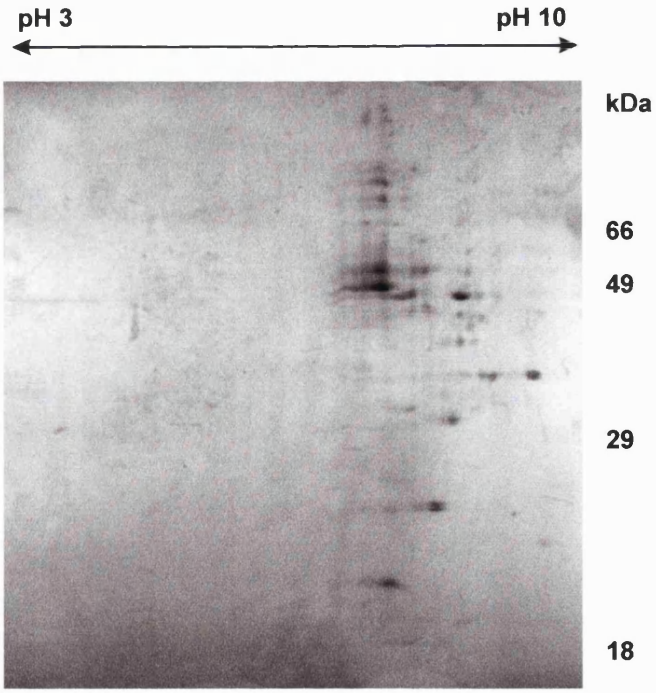

Figure 3.3 Two-dimensional SDS-PAGE analysis of the protein composition of the EM of *S. sanguis* grown in the presence of blood. Proteins were separated in the first dimension on the basis of their isoelectric point, and in the other dimension on the basis of their molecular mass on a 12% gel. The gel was stained with silver stain to visualise proteins. The positions of the molecular mass markers are shown on the right-hand side.

3.3.3.2 Detection of contaminating proteins from horse blood in EM

EM from *S. sanguis* grown in the presence of blood was separated by SDS-PAGE and electroblotted onto nitrocellulose membrane. Horse serum prepared from whole horse blood (positive control) and an *E. coli* lysate (negative control) were run alongside the EM. No bands were detected in the negative control when the blot was probed with anti-whole horse serum, but both the positive control and the *S. sanguis* EM showed clear bands (figure 3.4). The two major proteins detected in the EM were approximately 61 and 27 kDa in size, corresponding closely to the calculated mass of the two predominant proteins detected in the horse serum control lanes: 63 and 26 kDa.

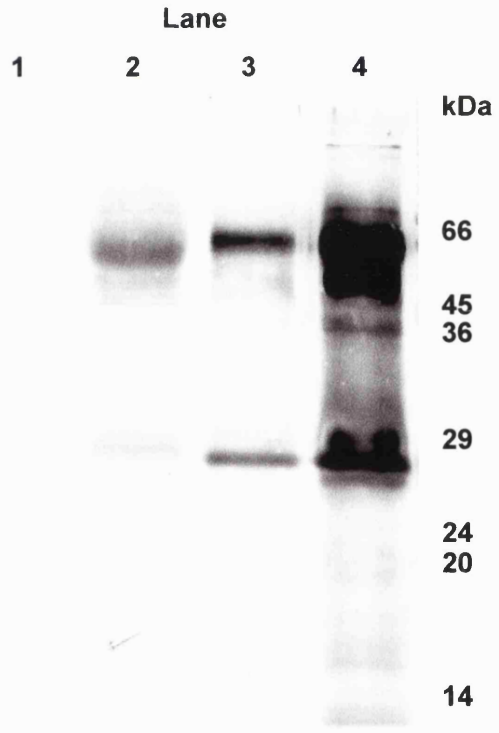

Figure 3.4 Western blot analysis of blood-grown *S. sanguis* EM. Proteins were separated on 12% gels and electroblotted using the Tris-glycine buffer system. Immunoblots were probed with a 1/1000 dilution of anti-whole horse serum (Sigma). Secondary antibody (Goat-anti-rabbit-HRP conjugate (Sigma)) was used at a 1/500 dilution. Lane 1 shows the negative control (*E. coli* lysate); lane 2 shows 7.5μg *S. sanguis* EM (equivalent to 1.5μg protein); lane 3 shows a 1/10,000 dilution of horse serum; lane 4 shows a 1/1000 dilution of horse serum.

3.4 Discussion

The rationale for this thesis is that extracellular molecules of *S. sanguis* may be involved in bacterial-host cell communication by the modulation of the cytokine response of host cells. The work presented in this chapter was aimed at isolating and characterising the extracellular material of *S. sanguis*, grown on agar plates rather than in liquid culture as an approximation of biofilm growth.

Ruthenium red is a dye used to stain mucopolysaccharides (polysaccharides containing a high proportion of amino sugars and uronic acids, such as hyaluronic acid). It detects acidic polymers, with the reactive component being either an acidic sugar or a polysaccharide. Mills and colleagues used ruthenium red to detect the capsule or glycocalyx around streptococci, both *in vivo* and *in vitro* (Mills *et al.* 1984). In the study presented here, transmission electron microscopic examination of *S. sanguis* stained with ruthenium red before saline extraction, revealed a large amount of extracellular material, some of which was fibrillar in nature. This material was removed by a gentle extraction with saline.

It is often assumed that capsular material is comprised almost entirely of polysaccharide-containing molecules, but the studies of McNab and colleagues demonstrated the presence of polypeptides as an important constituent of the extracellular material of *S. gordonii* (McNab *et al.* 1996; McNab *et al.* 1999). With this in mind, the composition of the saline-extracted extracellular material was investigated, and found to contain a considerable amount of protein.

To give an indication of the variation between estimates of protein content measured by different methods, the Lowry assay and the dye-binding assay were performed in addition to the Bio-Rad DC assay. For one representative batch of EM, the three assays gave very different estimates of the protein content. The commercial Bio-Rad DC protein assay and the Lowry assay gave the most similar results: 24% and 18% respectively. Since the Bio-Rad DC assay is based on the same chemistry as the Lowry assay, primarily detecting tyrosine and tryptophan residues, this result is unsurprising.

The slightly higher protein content estimated by the Bio-Rad assay may have been because the kit is modified to be compatible with many chemicals (particularly detergents) which otherwise interfere with the Lowry reaction. In particular, agents that acidify the solution, chelate the copper, or cause the reduction of copper, are known to interfere with the Lowry assay. In contrast to the Lowry and Bio-Rad DC assays, the Bradford (or "Dye Binding") assay detects primarily basic amino acid residues, especially arginine, and to a lesser extent histidine, lysine, tyrosine, tryptophan and phenylalanine residues. This difference in the amino acids detected may explain the low estimate of protein in the EM by this method (2.4%) compared to the other two assays, if the proteins have a low arginine content. The underestimate may also have resulted from the use of BSA as the standard, as this tends to give a higher A_{595} value than most proteins.

For all subsequent measurements of protein, the Bio-Rad DC assay was used, as it proved to give reproducible results, and the microassay using microtitre plates reduced the amount of sample needed.

The estimate of carbohydrate content in the EM was very low, ranging from 3 to 4%. The Dubois carbohydrate assay could be used to detect molecules such as LTA, by virtue of their sugar backbone, but unless purified LTA was used to produce a standard curve, the amount present would be greatly underestimated. The extraction of lipid from 10mg of EM failed to yield enough to be accurately weighed, suggesting that EM could not have been more than about 5% lipid. The sum of the percentages of protein, carbohydrate, nucleic acid and lipid falls far short of 100% (approximately 35% at most), and it may be that macromolecules such as peptidoglycan and teichoic acids are underestimated by the assays used.

EM was shown to contain less than 0.00002% endotoxin by the Limulus amoebocyte assay. It may seem odd to have measured the amount of endotoxin present in material extracted from a Gram-positive organism. However, laboratory water from supposedly clean sources has been found to contain levels of endotoxin which border on

stimulatory in routine assays where IL-6 secretion from isolated PBMC is measured (unpublished findings at the Eastman Dental Institute). Where possible, sterile plasticware and pyrogen-free water was used throughout this study. Lipoteichoic acid, sometimes considered to be the Gram-positive equivalent of LPS, can also be detected by the Limulus assay, but is much less reactive than *E. coli* LPS, the standard used for this assay: Renzi and colleagues found that LTAs extracted from various Gram-positive organisms were 3 to 6 orders of magnitude less reactive in the LAL assay than *E. coli* LPS (Renzi and Lee, 1995). Preliminary experiments to compare the reactivity of a commercial preparation of LTA from *S. sanguis* (Sigma) with the *E. coli* LPS standard in the LAL assay, suggested that 3pg of LPS was of equivalent activity to between 40 and 80ng of *S. sanguis* LTA (data not shown). This would mean that LTA was between 13,000 and 26,000 times less potent than LPS in the LAL assay, a result that agrees with the findings of Renzi and co-workers. A concentration of LPS in EM of 0.00002% would thus translate to a concentration of between 0.26 and 0.52% LTA. However, these are only preliminary results and should be interpreted with caution as the commercial preparation of LTA was from a different strain of *S. sanguis* to that used for production of EM, and reactivity in the LAL assay may vary accordingly. The issue of contamination of EM with lipoteichoic acid will be addressed more fully in Chapter 4, in which results from studies concerning the biological activity of EM are presented.

Separation of the EM by one-dimensional SDS-PAGE revealed the presence of at least 30 polypeptides ranging in size from 14 to greater than 100kDa. Since one-dimensional SDS-PAGE separates polypeptides only on the basis of their molecular mass, different polypeptides of the same molecular mass may appear as a single band on a gel. To give a clearer indication of the number of polypeptides present, the EM was also separated by two-dimensional electrophoresis. This technique separates proteins according to their isoelectric point (the "first dimension") and their molecular mass (the "second dimension"), resulting in discrete protein spots. Using this method the EM was shown to contain between 45 and 50 distinct proteins, although the separation in the first dimension was disappointing. Several proteins did not focus completely during isoelectric focusing, and appeared as broad smears. The use of commercially produced

immobilised pH gradients (IPG strips) in the first dimension, rather than the mobile pH gradients used in this study, can improve focusing, but this technology was not available to me at the time.

The possibility that the EM contained components from horse blood stemmed from the knowledge that streptococci possess surface proteins that bind to elements in blood. Protein G binds to equine IgG, and has been used to purify equine IgG subisotypes (Sheoran and Holmes, 1996). To examine whether the EM from *S. sanguis* grown in the presence of blood contained contaminating proteins from the horse blood used to supplement the growth medium, EM was electroblotted and probed with anti-whole horse serum. The detection of two proteins of 27 and 61 kDa, corresponding approximately to the two major proteins detected in the control horse serum (26 and 63 kDa), suggested that EM did indeed contain components derived from the blood-supplemented growth medium. It is possible that the proteins detected in both the EM and the horse serum are the light and heavy chains of horse immunoglobulin G (IgG). The light chains of all equine IgG isotypes are 27kDa, while the heavy chains vary from 52kDa to 60kDa (under reducing conditions) (Sheoran and Holmes, 1996).

It would have been relatively straightforward to remove contaminating IgG from preparations of EM by means of a protein A affinity column. However, since this procedure would probably have resulted in loss of protein, an increased risk of LPS contamination occurring, and the molecular mass of the contaminants was known, it was decided not to carry this out.

This section of the study has shown that *S. sanguis* grown as a biofilm secretes extracellular material that can be removed by a gentle saline extraction, and that this material contains a large number of proteins. The following two chapters describe the biological activity of this material, and the attempts made to isolate the individual components responsible for this activity.

CHAPTER 4

CYTOKINE-MODULATING ACTIVITY OF *S. sanguis* EXPORTED MATERIAL (EM)

4.1 Introduction

Cytokines play a central role in inflammatory processes, and since infective endocarditis involves inflammatory pathology, they are likely to be important in the perpetuation of this disease. Many bacterial components are able to elicit a cytokine response from human cells, both immune cells and cells such as fibroblasts and endothelial cells (Wilson *et al.* 1996; Henderson *et al.* 1996a; Henderson and Wilson, 1996b). In bacteria associated with a solid endocardial vegetation, an important source of cytokine-inducing molecules is likely to be the soluble material secreted from such organisms, as this may be able to diffuse through the platelet-fibrin matrix and reach the incoming immune cells.

Soluble material found loosely-associated with the surface of several species of bacteria, previously termed "Surface-associated material" or "SAM", exhibits a variety of biological activities, including the ability to induce cytokine secretion from a number of different human cell types. SAM from organisms implicated in the periodontal diseases such as *A. actinomycetemcomitans* and *Por. gingivalis* has been shown to stimulate human gingival fibroblasts (HGFs) and human PBMCs to produce the inflammatory cytokines IL-1β, IL-6 and TNFα (Reddi *et al.* 1996b). Exported material from both *Staph. aureus* and *Staph. epidermidis* has been demonstrated to possess cytokine-modulating properties (Crean *et al.* 1997; Crean *et al.* 1998)

In this part of the study, the cytokine-inducing activity of the soluble, exported material (EM) from *S. sanguis* has been investigated, and attempts have been made to characterise the type of molecules that may be responsible for such activity. The effect of EM on mRNA for more than 200 cytokine-related proteins in PBMCs has also been assessed using a commercial cDNA array.

4.2 Materials and Methods

4.2.1 Stimulation of human PBMCs with EM isolated from *S. sanguis* grown in the presence of blood

PBMCs were isolated from single-donor buffy coat blood and dispensed into 24-well tissue culture plates at 2 x 10^6 cells/ml as described in Chapter 2, sections 2.5.1 and 2.5.2. Freeze-dried *S. sanguis* EM (prepared as described in Chapter 3, section 3.2.2) was dissolved in pyrogen-free water, dispensed into sterile 1.5ml Eppendorfs, and centrifuged in an Eppendorf 5810R centrifuge with an FA-45-30-11 rotor for 10 minutes at 14,000 x g (4°C) to remove undissolved particulates. A series of dilutions of solubilised EM was prepared in sterile PBS, and the appropriate amounts added in triplicate to the 24-well culture plates containing PBMCs. As a positive control *E. coli* lipopolysaccharide (LPS; Difco, UK) was added to give a final concentration in the assay wells of 10ng/ml. Plates were incubated for 16 hours before harvesting the cell culture supernatants and assaying for IL-1β, IL-6, IL-8 and TNFα, as described in Chapter 2, section 2.6.

4.2.2 Assessment of the effect of heat and trypsin digestion on the cytokine-inducing activity of *S. sanguis* EM

4.2.2.1 Trypsin digestion of *S. sanguis* EM

Freeze-dried EM was dissolved in 50mM Tris pH 7.6 prepared with pyrogen-free water to a concentration of 1mg/ml EM (equivalent to 0.125mg/ml protein), and centrifuged to remove particulates as described in section 4.2.1. Two aliquots of EM were retained, one for heat treatment (see section 4.2.2.2 below) and the other to serve as an untreated control. A stock solution of sequencing grade trypsin (Sigma) at 50μg/ml in 0.01% trifluoroacetic acid was added to an aliquot of solubilised EM to give a final concentration of trypsin of 2.5μg/ml, and a final concentration of EM of 0.95mg/ml (equivalent to 0.119mg/ml protein). This gave a ratio (w/w) of trypsin/protein of 1/48, which was within the recommended dilution limits stated by the manufacturer. A control sample was also prepared, identical to the experimental reaction mixture except

for the inclusion of 50mM Tris pH 7.6 in place of the solubilised EM. Both were incubated for 5 hours at 37°C. The trypsin digestion was stopped by the addition of the irreversible protease inhibitor PMSF (stock of 200mM in ethanol) to give a final concentration in the reaction mix of 1mM, and incubation for 2 minutes at 37°C. The PMSF itself was inactivated by incubating the samples for at least 1 hour at room temperature, and by further dilution on addition of the samples to the PBMC. Samples were tested in a routine PBMC assay (section 2.5) and assayed for the induction of IL-1β, IL-6 and TNFα (section 2.6).

4.2.2.2 Heat-treatment of *S. sanguis* EM

An aliquot of EM that had been prepared for the trypsin digestion (see section 4.2.2.1 above) was used for the heat-inactivation experiment. Solubilised EM was dispensed into sterile Eppendorfs and the lids sealed in place with parafilm before heating in a boiling water-bath for 30 minutes. Samples were allowed to cool to room temperature before testing in the routine PBMC assay, and assayed for IL-1β, IL-6 and TNFα, as described in Chapter 2, sections 2.5 and 2.6.

4.2.3 Optimisation of commercial preparations of *E. coli* LPS and *S. sanguis* lipoteichoic acid (LTA) for use in PBMC assays

E. coli LPS (Difco, UK) and *S. sanguis* LTA (Sigma) were tested in routine PBMC assays as described in Chapter 2, section 2.5.2. LPS (5mg/ml stock solution stored in aliquots at -20°C) was diluted in pyrogen-free water (Associates of Cape Cod) and tested in triplicate at 0.16, 0.31, 0.63, 1.25, 2.5, 5 or 10ng/ml. In a separate set of experiments to look more closely at the effects of lower concentrations, LPS was tested at 25, 50, 100, 200, 400 and 800pg/ml. LTA was dissolved in pyrogen-free water and tested in triplicate at 0.001, 0.01, 0.1, 1, 10 and 100μg/ml. The remaining LTA was stored as a 10mg/ml stock at -20°C in 50μl aliquots.

4.2.4 Assessment of the effect of polymyxin B on the cytokine-inducing activity of *S. sanguis* EM

For the first set of experiments, PBMC assays were carried out as described in section 4.2.1, except that selected wells were incubated for 30 minutes with fresh medium containing polymyxin B (Sigma) at 20μg/ml, prior to the addition of stimuli. All other wells were incubated with fresh medium without polymyxin B. *S. sanguis* EM was tested at 0.03, 0.3, 3, 30 or 300μg/ml. To confirm the blocking activity of the antibiotic, controls of *E. coli* LPS at 10ng/ml were also tested in the presence and absence of 20μg/ml polymyxin B. Levels of IL-1β, IL-6 and TNFα in the supernatants were measured by ELISAs as described in Chapter 2, section 2.6.

In a second set of experiments, the effect of an increasing concentration of polymyxin B on the cytokine-inducing activity of a fixed concentration *of S. sanguis* EM was assessed. Selected wells of PBMC were incubated for 30 minutes with fresh medium containing 0, 1.25, 2.5, 5.0, 10, or 20μg/ml polymyxin B immediately prior to the addition of the stimuli. *S. sanguis* EM was added to the wells to give a final concentration of 12μg/ml (a concentration shown to be sub-maximal for this preparation of EM - see Figure 4.4). To serve as controls, fixed concentrations of *E. coli* LPS (10ng/ml) and *S. sanguis* LTA (100ng/ml) (Sigma) were also tested in the presence of the same range of concentrations of polymyxin B. Cells cultured in polymyxin B-containing medium, but without a stimulating agent were also included to control for the effect of the antibiotic alone on cytokine production by PBMC.

4.2.4.1 Assessment of the blocking effect of polymyxin B on various concentrations of LPS and LTA

A routine PBMC assay was carried out as described in section 2.5.2, with selected wells of PBMC incubated with culture medium containing 20μg/ml polymyxin B for 30 minutes prior to the addition of stimuli. *E. coli* LPS was added at 50, 100, 150, 200, 250 or 300ng/ml, to triplicate wells of PBMC incubated both with and without polymyxin B. *S. sanguis* LTA at 10, 20, 30, 40 or 50μg/ml, was added to PBMC

cultured both with and without polymyxin B. Supernatants were assayed for IL-6 (Chapter 2, section 2.6).

4.2.5 Assessment of the effect of antibodies against CD14 on the cytokine-inducing activity of *S. sanguis* EM

The anti-CD14 antibody 60bca, prepared from the mouse hybridoma cell line HB-247 (ATCC), was kindly provided by Dr Jo Lewthwaite (Eastman Dental Institute). The ability of both hybridoma supernatant and affinity-purified 60bca to block LPS-induced IL-6 secretion from PBMC was assessed (see Chapter 2, sections 2.5 and 2.6 for details of the routine PBMC assay and ELISA). The optimum concentration of purified 60bca for blocking LPS activity was found to be 15μg/ml in a PBMC assay using 10ng/ml LPS (work carried out by Dr. Jo Lewthwaite - data not shown).

4.2.5.1 Assessment of the effect of hybridoma supernatant containing anti-CD14 antibody on the cytokine-inducing activity of *S. sanguis* EM

PBMC were prepared as described in Chapter 2, section 2.5. After the adherence and washing step, the culture medium was replaced with either fresh culture medium or undiluted hybridoma supernatant. *S. sanguis* EM was tested in triplicate at 0.03, 0.3, 3, 30 or 300μg/ml, both in the presence and absence of hybridoma supernatant. As a positive control for the blocking effect of the hybridoma supernatant, LPS at 10ng/ml was also tested.

4.2.5.2 Assessment of the effect of purified 60bca on the cytokine-inducing activity of *S. sanguis* EM

PBMC were prepared as described in Chapter 2, section 2.5. After the adherence and washing step, the culture medium was replaced with either fresh culture medium, or culture medium containing 3.75, 7.5 or 15μg/ml purified 60bca. PBMC were incubated with the blocking antibodies for 1 hour prior to the addition of stimuli. *E. coli* LPS (10ng/ml), or *S. sanguis* EM (2μg/ml), was tested in triplicate, either alone, or in the presence of the various dilutions of purified antibodies described above. After 16 hours

incubation, PBMC culture supernatants were assayed for IL-6 by ELISA (Chapter 2, section 2.6).

4.2.5.3 Assessment of the effect of the anti-CD14 monoclonal antibodies 60bca or MY4 on the cytokine-inducing activity of *S. sanguis* EM

These experiments were carried out as described in section 4.2.5.2 above. PBMC were pre-incubated with purified 60bca or its isotype-matched control mAb (mouse IgG1, Sigma) at 7.5 and 15μg/ml. MY4 (Beckman Coulter, UK) or its isotype-matched control MAb (IgG2b, Sigma) was used at 5 and 10μg/ml. *S. sanguis* EM was added to PBMC to give a final concentration of 2μg/ml. As a positive control for the blocking effect of both 60bca and MY4, *E. coli* LPS was also tested at 10ng/ml. PBMC were incubated for 16 hours before assaying the supernatants for IL-6 as described in Chapter 2, section 2.6.

4.2.6 Time course of cytokine production by PBMC in response to *S. sanguis* EM

A routine PBMC assay was carried out (Chapter 2, section 2.5.1), with one 24-well plate used for each time-point. *E. coli* LPS was tested at 10ng/ml and *S. sanguis* EM was tested at 20μg/ml. For each time-point (0,1,2,4 or 8 hours) control wells of unstimulated PBMC were included to assess background levels of cytokine production. Levels of IL-1β, IL-6, IL-8 and TNFα in the culture supernatants were measured by ELISAs, details of which are given in Chapter 2, section 2.6. In a second experiment the time was extended to include 20 and 24 hours post-stimulation to ascertain the time-point at which cytokine production by PBMC had reached a plateau. After the collection of culture supernatants for ELISAs, the PBMC from the second experiment were subjected to RNA extraction in preparation for Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) to examine cytokine mRNA production (see section 4.2.7 below).

4.2.7 Time course of cytokine mRNA production in PBMC stimulated with *S. sanguis* EM

Culture supernatants were removed from the wells and 25µl of a 400µg/ml stock of tRNA (Sigma) was added to the first of each set of triplicate wells. 200µl of solution D (4M guanidinium thiocyanate (Sigma), 25mM sodium citrate pH 7.0, 0.5% sodium sarcosyl, 0.75% 2-mercaptoethanol (Sigma)) was added to each well. Cells were scraped off the wells, and the pooled triplicates transferred to sterile 1.5ml Eppendorfs before being frozen at -70°C.

4.2.7.1 RNA extraction

RNA extraction was carried out using an adaptation of the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Samples were defrosted before adding the following to each tube:-

50µl 3M sodium acetate pH 5.0 (Sigma)
500µl citrate-saturated phenol pH 4.3 (Sigma) + 0.05% 8-hydroxyquinoline
100µl chloroform:isoamyl alcohol (49:1; Sigma)

Tubes were vortexed for 10 seconds to ensure complete homogenisation and then incubated on ice for 10 minutes, before being centrifuged at 4°C for 10 minutes at 14,000 x g in an Eppendorf 5810R centrifuge with an FA-45-30-11 rotor. 500µl from the top aqueous layer was transferred to a fresh Eppendorf, to which an equal volume of isopropanol was added. Tubes were vortexed thoroughly and incubated overnight at -20°C to precipitate the RNA. Samples were centrifuged for 30 minutes at 14,000 x g as described above, and the supernatant discarded. Pellets of precipitated RNA were dissolved in 150µl solution D (see 4.2.7). This was followed by the addition of 150µl isopropanol to reprecipitate the RNA. Tubes were vortexed and then incubated for 1-2 hours at -20°C. Precipitates were pelleted by centrifugation for 30 minutes at 14,000 x g as described above, and the supernatants discarded. Pellets were washed with 250µl

of 80% ethanol and centrifuged for 10 minutes before discarding the supernatants and allowing the pellets to air dry.

4.2.7.2 Generation of cDNA

Each RNA pellet was dissolved in 15µl Diethylpyrocarbonate (DEPC) -treated water plus 1µl polythymidine (oligo dT) (Sigma-Genosys) and heated to 70°C for 10 minutes to denature the RNA. Tubes were placed on ice before adding 4µl of 5 x first strand buffer (Gibco BRL), 2µl of 0.1M DTT (Gibco BRL) and 1µl dNTPs (stock containing 10mM of each dNTP; Amersham Pharmacia Biotech) to each, and heating at 42°C for 2 minutes. While still at 42°C, 0.5µl of Superscript II Reverse Transcriptase (Gibco BRL) and 1.5µl DEPC water was added to each sample before incubating for a further one hour. Samples were heated at 70°C for 10-15 minutes to stop the reaction, and then stored at -20°C until the PCR was carried out.

4.2.7.3 PCR

The protocols for all PCR reactions were as described by Reddi et al (1996). The cDNA was diluted 1 in 4 with DEPC water and 5µl aliquots transferred to 0.5ml PCR tubes (Anachem, UK). A negative control of DEPC water, and a positive control of an appropriate PCR product diluted 1 in 40 with DEPC water (both of 5µl volume), were included. The following was added to each tube:-

Solution	**Volume added per tube (µl)**
10 x PCR buffer (Gibco BRL)	5
50mM $MgCl_2$ (Gibco BRL)	1.5
dNTPs (10mM each)	1
Forward primer (10µM)	0.5
Reverse primer (10µM)	0.5
Taq polymerase 5 units/µl (Gibco BRL)	0.25
DEPC water	36.25

Total volume **45**

The following primers (all from Sigma-Genosys) were used:-

GAPDH forward - 5'-CCACCCATGGCAAATTCCATGGCA-3'
GAPDH reverse - 5'-TCTAGACGGCAGGTCAGGTCCACC-3'

IL-1β forward - 5'-AAACAGATGAAGTGCTCCTTCCAGG-3'
IL-1β reverse - 5'-TGGAGAACACCACTTGTTGCTCCA-3'

IL-6 forward - 5'-ATGAACTCCTTCTCCACAAGCGC-3'
IL-6 reverse - 5'-GAAGAGCCCTCAGGCTGGACTG-3'

IL-8 forward - 5'-ATGACTTCCAAGCTGGCCGTGGCT-3'
IL-8 reverse - 5'-TCTCAGCCCTCTTCAAAAACTTCTC-3'

TNFα forward - 5'-CGGGACGTGGAGCTGGCCGAGGAG-3'
TNFα reverse - 5'-CACCAGCTGGTTATCTCTCAGCTC-3'

The following programs were run using an Eppendorf Mastercycler 5330 PCR machine:-

All PCR protocols were performed with a 3 minute denaturation step at 96°C. The temperature profile of the amplification for GAPDH consisted of 30 cycles of 45 seconds at 94°C, 1 minute at 60°C, 1.5 minutes at 72°C and a final extension time of 10 minutes at 72°C. For IL-1β, IL-6 and IL-8, each cycle consisted of 1 minute at 95°C followed by 2.5 minutes at 60°C (annealing and extension were carried out at the same temperature), with 30, 30 and 25 cycles performed, respectively. For TNFα,

amplification was carried out as for IL-1β, IL-6 and IL-8 except that annealing and extension were carried out at 72°C, and that 35 cycles were performed.

12.5µl of sample buffer (30% sterile glycerol in autoclaved dH_2O, plus 200µg/ml bromophenol blue (Sigma)) was added to each tube of PCR product and 20µl run on Tris-borate EDTA agarose gels. Gels were 1% agarose (Bioline, UK) in Tris-borate EDTA buffer (0.045M Tris-borate, 0.001M EDTA), with 0.5µg/ml ethidium bromide (Sigma). 7µl of a 100bp DNA ladder (Promega, UK) was run as a marker lane. Tris-borate EDTA buffer was used as the running buffer. Gels were run at 100 volts for 30-60 minutes on a Gibco Horizon 11.14 system, and photographed under UV light using a UVP image analysis system.

4.2.8 Assessment of the effect of *S. sanguis* EM on cytokine gene transcription patterns using a commercial cDNA array

PBMC were prepared as described in Chapter 2 section 2.5.1, and plated out at $2x10^6$/ml into four 6-well plates (4.75ml of cells/well). Cells were allowed to adhere for 1 hour before the wells were washed once with PBS and the culture medium replaced. Two 6-well plates were stimulated with *S. sanguis* EM at 2µg/ml; the remaining plates were left as unstimulated controls. After an 8 hour incubation, the plates were centrifuged for 15 minutes at 300 x g, and the remaining culture medium discarded. 600µl of solution D (see section 4.2.7) was added to each well and the plates transferred immediately to –70°C.

4.2.8.1 RNA extraction

Plates were allowed to thaw and the lysed cells scraped from the wells before being transferred to fresh 1.5ml Eppendorfs (1 tube per well). RNA extraction was carried out as described in section 4.2.7.1, except that the pellets from each experimental condition obtained after the first isopropanol precipitation were pooled. At the end of the extraction procedure the air-dried pellets were resuspended in 250µl RNase-free water (Clontech, UK). A small portion of each sample (5µl) was diluted 1 in 20 with

RNase-free water, and the concentration of RNA measured in a spectrophotometer (at 260nm) (Pharmacia, UK). The remaining RNA was stored at -70°C.

4.2.8.2 DNase treatment of total RNA

The RNA was thawed and RNasin (Promega) was added to each tube to a final concentration of 1 unit/µl. All other reagents used were from Clontech. The following reaction mix was prepared for each RNA sample:-

Total RNA (0.3 mg/ml)	250µl
10 x DNase I Buffer	40µl
DNase I (at 1 unit/ml)	20µl
RNase-free water	140µl
Total volume	450µl

Samples were incubated at 37°C for 30 minutes before terminating the reaction by the addition of 50µl of 10 x Termination Mix (Clontech). 500µl of saturated phenol and 300µl chloroform were added to each tube and vortexed thoroughly. Samples were centrifuged at 14,000 x g for 10 minutes at 4°C. The top aqueous layer was transferred to a fresh Eppendorf to which 550µl of chloroform was added, before centrifuging as described previously to separate the phases. The top phase was collected and 1/10 the volume of 2M sodium acetate pH4.5 and 2.5 volumes of 95% ethanol were added. Tubes were vortexed thoroughly and incubated for 1 hour at -20°C. Precipitates were recovered by centrifugation at 14,000 x g for 15 minutes at 4°C, and the supernatants discarded. Pellets were washed with 250µl of 80% ethanol and centrifuged for 15 minutes as described above. After air-drying, the pellets were redissolved in 50µl of RNase-free water. Five microlitres from each sample was used to determine the concentration and total amount of RNA as described previously. The remaining RNA was stored at -70°C.

4.2.8.3 Enrichment of poly A-tailed mRNA and probe synthesis

Thirty microlitres of streptavidin-coated magnetic beads (15µl per probe synthesis reaction) were prepared by washing four times with 400µl of 1 x binding buffer (Clontech), using a Dynex magnet to recover the beads. The beads were resuspended in 30µl of 1 x binding buffer.

10µg of total RNA from each sample was dispensed into a 0.5ml Eppendorf and the volume made up to 45µl with RNase-free water. One microlitre of biotinylated Oligo(dT) was added to each sample and incubated for 2 minutes at 70°C using a thermal cycler (Eppendorf). Samples were allowed to cool at room temperature for 10 minutes before adding 45µl of 2 x binding buffer to each tube. 15µl of resuspended magnetic beads were added to each sample. The tubes were then incubated at room temperature for 25 minutes on a vortex mixer to ensure the continual mixing of the beads. The beads were recovered using the magnet and washed twice with 50µl of 1 x wash buffer, followed by a single wash with 50µl of 1 x reaction buffer. After discarding the reaction buffer, the beads were resuspended in 6µl of RNase-free water.

4.2.8.4 cDNA probe synthesis

This was kindly carried out by Dr Peter Tabona (Eastman Dental Institute). A Master Mix was prepared at room temperature allowing enough volume for one extra reaction:-

	Volume added per sample (μl)
5 x Reaction Buffer	4μl
10 x dNTP Mix (5mM each of dCTP, dGTP and dTTP)	2μl
[α-^{32}P]dATP (3000 Ci/mmol, 10μCi/μl)	5μl
DTT (100mM)	0.5μl
MMLV Reverse Transcriptase*	2μl
Total volume	**13.5μl**

*The MMLV Reverse Transcriptase was added just prior to the addition of the Master Mix to the beads.

One microlitre of Primer mix (Clontech) was added to the 6μl of resuspended beads. Tubes were incubated at 65°C for 2 minutes in a thermal cycler, followed by a further 2 minutes at 50°C. Thirteen and a half microlitres of Master Mix was added to each tube and then incubated at 50°C for 25 minutes. The reaction was terminated by the addition of 2μl Termination Mix and incubation at 70°C for 5 minutes. The probes were purified from unincorporated ^{32}P-labelled nucleotides and small (<0.1kb) cDNA fragments by column chromatography using Chromaspin columns (Clontech) in accordance with the manufacturers instructions. Fractions were collected and assessed for the level of ^{32}P using a Wallac 1409 liquid scintillation counter (EG & G Wallac, UK). The fractions containing the first peak of activity (approximately 5-10%) were pooled, and stored at -70°C. The second peak, containing unincorporated ^{32}P-labelled nucleotides, and the remaining fractions were discarded appropriately.

4.2.8.5 Hybridizing cDNA probes to the Atlas cDNA array

All prehybridization, hybridization and washing steps were carried out in roller bottles, rotated at 5-7 rpm at 68°C in a Hybaid hybridisation oven. Sheared salmon testes DNA

(100µl of a 10mg/ml stock (Sigma)) was heated to 95°C for 5 minutes and then chilled quickly on ice before adding to 10 ml ExpressHyb solution, prewarmed to 68°C. This solution was kept at 68°C until use. Each Atlas Array membrane was prepared for prehybridization by incubating for 2 minutes in 200ml of 0.5% SDS (pre-heated to 80-90°C and then removed from heat before adding the membranes). Membranes were then transferred to hybridization bottles (Biometra) and rinsed once with dH_2O before prehybridizing for 30 minutes at 68°C with 5ml of the ExpressHyb/salmon testes DNA solution. Labelled probes (both containing approximately the same amounts of radioactivity) were denatured by boiling for 2 minutes, cooled on ice for 2 minutes, and then added to the prehybridization solution in the roller bottle (not directly poured onto the membrane surface). Hybridization was carried out overnight with continuous agitation.

4.2.8.6 Development

The hybridization solution was discarded and the membranes washed four times with 200ml of wash solution 1 (0.3M NaCl, 0.03M trisodium citrate pH 7.0, 1% SDS, prewarmed to 68°C) for 30 minutes, followed by a single 30 minute wash with 200ml of wash solution 2 (15mM NaCl, 1.5mM trisodium citrate pH 7.0, 0.5% SDS, prewarmed to 68°C). One final 5 minute wash with 200ml of wash solution 1 was carried out. The membranes were wrapped in Saranwrap and exposed to x-ray film (Kodak, UK) at -70°C for various lengths of time (overnight, 3 days (66 hours), and 7 days (168 hours)).

4.3 Results

4.3.1 Cytokine-inducing activity of *S. sanguis* EM

Incubation with *S. sanguis* EM induced human PBMC to secrete all four of the pro-inflammatory cytokines assayed: IL-1β, IL-6, IL-8 and TNFα (figure 4.1). Activity was compared with the effect of 10ng/ml *E. coli* LPS, a concentration that consistently gives a maximal response from PBMC cultured at this density (data not shown). The response to the EM was dose-dependent for all four cytokines, although only levels of IL-8 and TNFα continued to increase with concentrations of EM greater than 30µg/ml;

the level of IL-6 did not increase significantly between 30 and 300μg/ml EM, and the production of IL-1β actually decreased between the same concentrations of EM. IL-8 was induced by 0.03μg/ml EM, while PBMC required 0.3μg/ml EM to induce secretion of IL-1β, IL-6 and TNFα. For all four cytokines, induction of cytokine secretion began at concentrations of EM between 0.03 and 0.3μg/ml. The EM-induced secretion of IL-1β, IL-8 and TNFα exceeded the maximum level obtainable with LPS, and this was most striking for IL-8: the level of IL-8 induced by the EM reached approximately 500ng/ml, while LPS resulted in the secretion of less than 200ng/ml.

4.3.2 Effect of heat and trypsin digestion on the cytokine-inducing activity of *S. sanguis* EM

Heating the EM for 30 minutes in a boiling water-bath decreased the IL-6-inducing activity by 82% ($p<0.0001$), while digestion with trypsin decreased activity by 92% ($p<0.0001$) (figure 4.2a). The trypsin control, which contained all constituents except for the EM, did not induce significant IL-6 production by PBMC. Further study of heat-inactivation assessed the effect on the ability of EM to induce PBMC to produce IL-1β and TNFα secretion in addition to IL-6 (figure 4.2b). The level of IL-6 produced in response to heat-treated EM was 95% lower than that produced in response to untreated EM ($p<0.001$). Similar decreases in EM activity were observed for the induction of IL-1β and TNFα production (96 and 91% respectively; $p<0.001$).

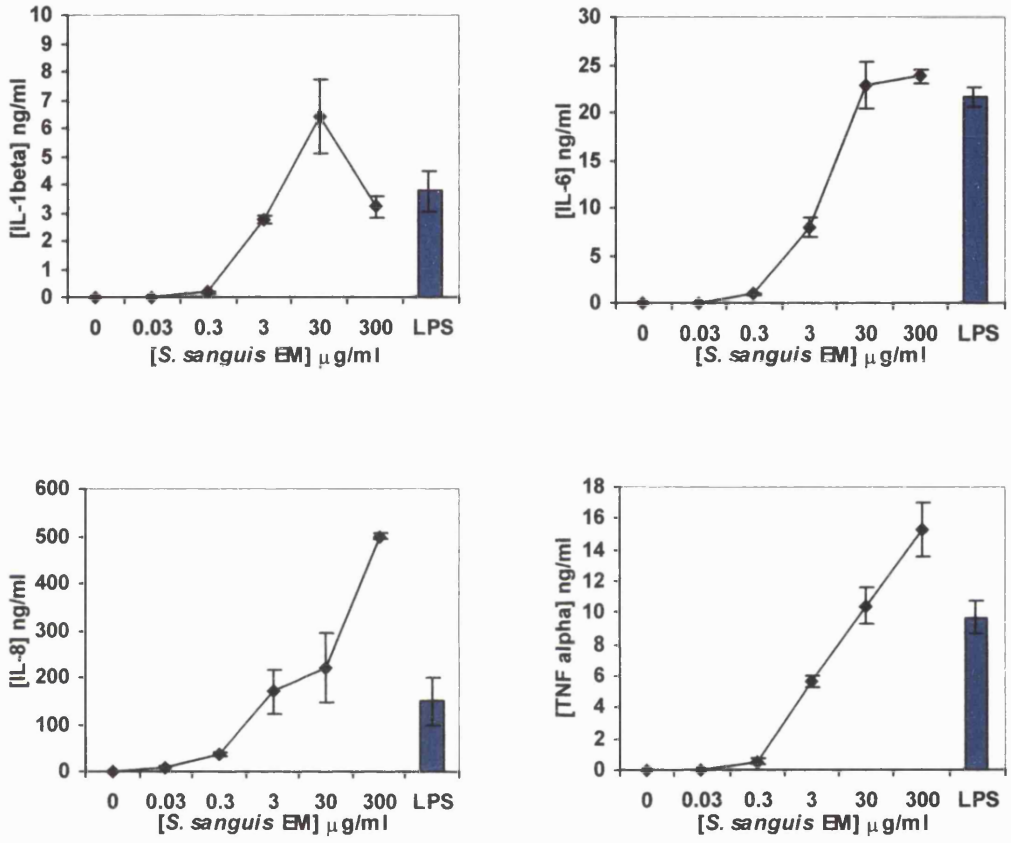

Figure 4.1 Stimulation of human PBMC with *S. sanguis* exported material (EM). Results shown are from a single representative PBMC preparation and are expressed as the mean and SD of triplicate cultures. Various concentrations of *S. sanguis* exported material were added to 1ml culture wells containing 2 x 10^6 cells. A positive control of *E. coli* LPS at 10ng/ml was included (shown as solid bars). After incubation for 16 hours the level of IL-1β, IL-6, IL-8 and TNFα in the cell supernatants was measured by ELISA.

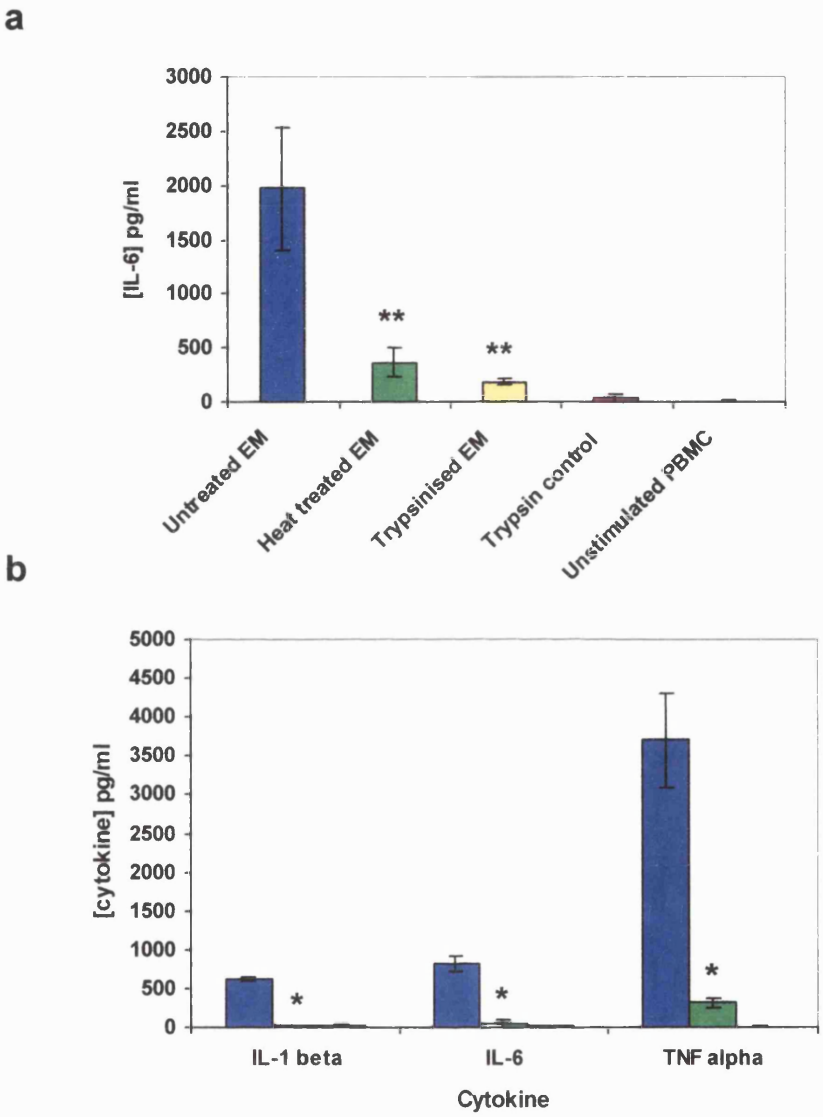

Figure 4.2 Effect of heating or trypsin digestion on the cytokine-inducing activity of *S. sanguis* EM. All results are expressed as the mean and SD of triplicate cultures. Panel (a) shows results from a single representative PBMC preparation. Exported material was heat-inactivated by boiling for 30 minutes. Sequencing-grade trypsin in 50mM Tris pH 7.6 was used for the trypsin digestion, with PMSF used to terminate the reaction. Panel (b) shows results from a separate experiment in which the effect of heating EM on IL-1β, IL-6 and TNFα production were assessed. Blue bars represent untreated EM; green bars represent heat-treated EM; yellow bars represent unstimulated PBMCs. *$p<0.001$ and **$p<0.0001$ when the level of cytokine produced in response to heat- or trypsin-treated EM is compared to that produced in response to untreated EM using a Students t-Test.

4.3.3 Optimisation of commercial preparations of *E. coli* LPS and *S. sanguis* lipoteichoic acid (LTA) for use in PBMC assays

Although considerable variation was observed in response to LPS in the maximum level of IL-6 produced by PBMC from different donors, 10ng/ml proved to give a consistent maximal response in all donors (Figure 4.3a). At lower concentrations of LPS, differences were also observed in the potency of LPS for PBMC isolated from different donors, with IL-6 secretion reaching a maximum at just 50pg/ml LPS in one donor, but requiring as much as 400pg/ml LPS to reach a plateau of IL-6 secretion in another (Figure 4.3b). In addition to IL-6, supernatants from PBMC stimulated with various concentrations of LPS were also assayed for IL-8, and concentrations of LPS above 0.16ng/ml were found to be maximal for induction of this cytokine (Figure 4.4). The commercial preparation of *S. sanguis* LTA induced PBMC to secrete IL-6, IL-1β and TNFα at concentrations greater than 0.01μg/ml, with maximal cytokine secretion occurring at between 1 and 10μg/ml LTA (Figure 4.5). IL-1β and IL-6 were secreted in the largest amounts, reaching approximately 25ng/ml in the culture supernatant in response to 10μg/ml LTA. However, the LTA appeared to be more potent in inducing IL-6 than either IL-1β or TNFα secretion.

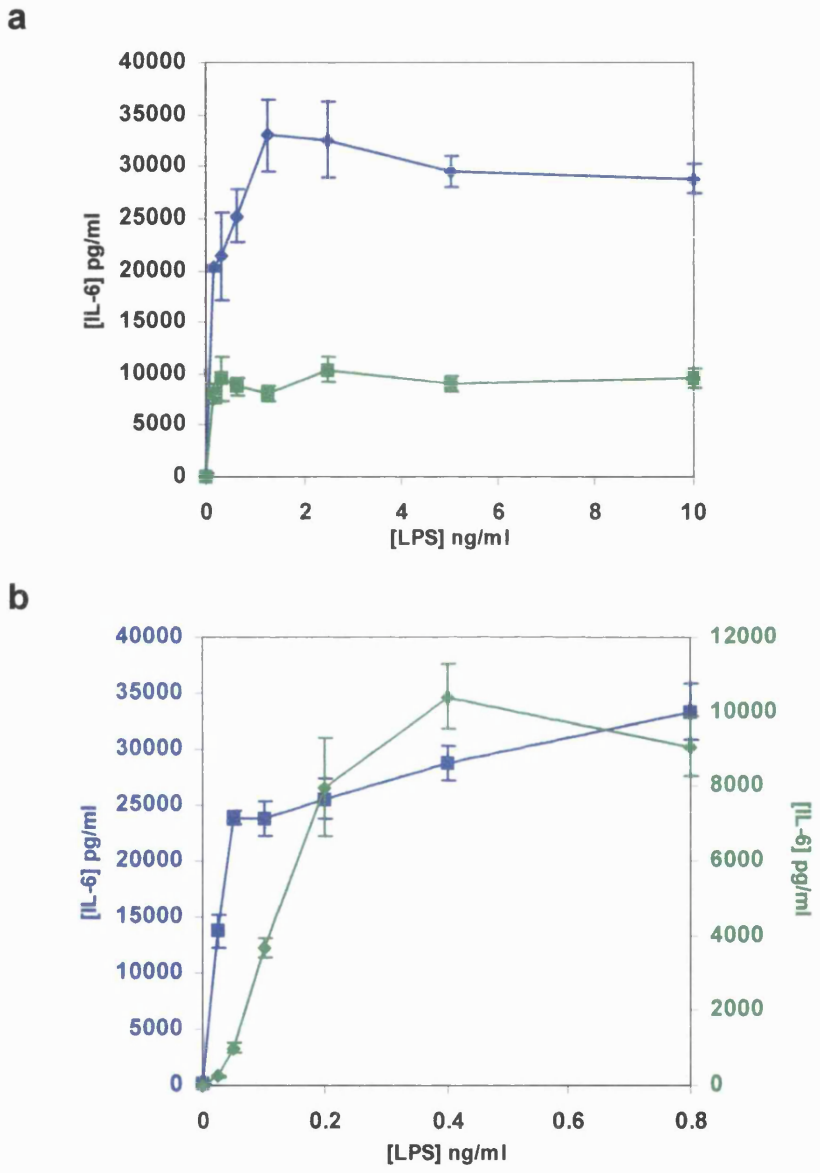

Figure 4.3 Stimulation of PBMC from four different donors with various concentrations of *E. coli* LPS. In each experiment, one donor is represented by the blue line and the second donor by the green line. Panel (a) shows the response to LPS of PBMC from two different donors, showing 10ng/ml LPS to be maximal for both donors, despite differing in the amounts of IL-6 produced. Panel (b) shows the response to LPS of PBMC from another two donors, focusing on lower concentrations of LPS, to illustrate differences in the potency of LPS depending on the donor. For easier comparison in panel (b), the results from the second donor (whose levels of IL-6 produced in response to LPS were generally lower than those seen for the first donor) have been plotted on a separate axis (green, right hand axis). Results are represented as the mean and SD of triplicate samples.

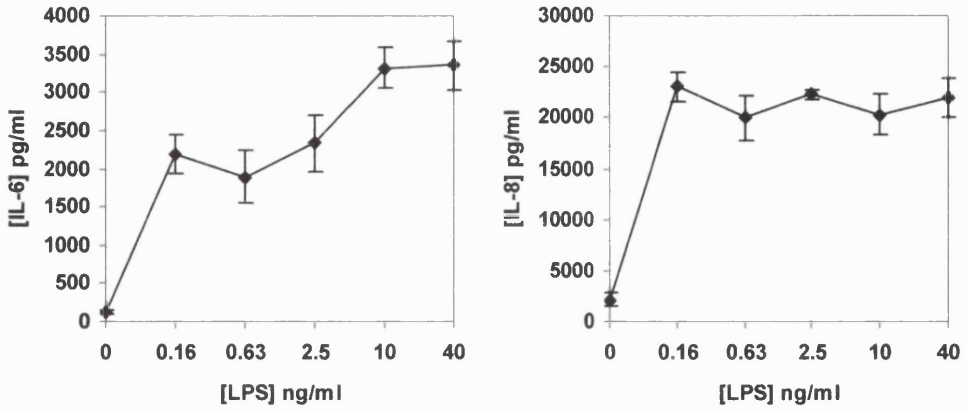

Figure 4.4 Stimulation of PBMC with various concentrations of *E. coli* LPS. Results shown are from a single representative PBMC preparation and are expressed as the mean and SD of triplicate samples. Various amounts of LPS were added to 1ml culture wells containing 2 x 10^6 cells. After incubation for 16 hours the level of IL-6 and IL-8 in the culture supernatants was measured by two-site ELISAs.

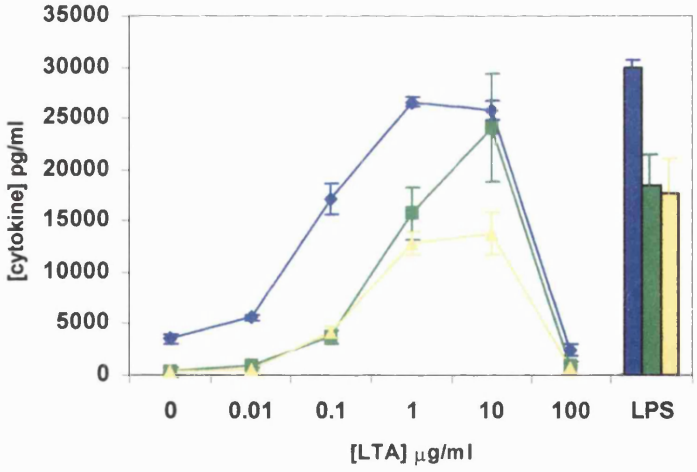

Figure 4.5 Stimulation of PBMC with various concentrations of *S. sanguis* LTA. Various amounts of LTA were added to 1ml culture wells containing 2 x 10^6 cells. Positive controls of LPS at 10ng/ml were also included. After incubation for 16 hours the amounts of IL-1β, IL-6 and TNFα in the culture supernatants were measured by two-site ELISAs. Results are expressed as the mean and SD of triplicate samples. Blue lines and bar represent IL-6, green lines and bar represent IL-1β and yellow lines and bar represent TNFα.

4.3.4 Effect of polymyxin B sulphate on the cytokine-inducing activity of *S. sanguis* EM

The addition of polymyxin B at 20μg/ml reduced the amount of IL-1β, IL-6 and TNFα produced by PBMC in response to 10ng/ml LPS to the level of the polymyxin B control (figure 4.6b, d and f). This confirmed the blocking activity of polymyxin B at this concentration. *S. sanguis* EM-induced IL-6 and TNFα were reduced in the presence of polymyxin B, but this appeared to be overcome at higher concentrations of EM (figure 4.6c and e). In contrast, polymyxin B significantly increased IL-1β production in response to the EM (for example, IL-1β secreted in response to 30μg/ml EM was increased by more than 300% ($p<0.01$ by a Students t-Test)) (figure 4.6a).

In a second set of experiments, secretion of IL-1β was again significantly increased when *S. sanguis* EM was tested in the presence of polymyxin B ($p<0.01$ for 2.5, 5 and 10μg/ml PB using a Dunnett's t-Test) (figure 4.7). The IL-6-inducing activity of the EM was not significantly affected by addition of polymyxin B. However, a significant increase in TNFα secretion was observed in response to the EM in the presence of 2.5 and 5μg/ml polymyxin B ($p<0.01$ and $p<0.05$ respectively using Dunnett's t-Test). At 20μg/ml polymyxin B, the level of TNFα secreted was not significantly lower than that observed in the absence of polymyxin B, although there did appear to be a downward trend. IL-1β, IL-6 and TNFα secretion in response to both the LPS and LTA controls was inhibited by polymyxin B in a dose-dependent manner. LPS-induced IL-1β was completely blocked by 5μg/ml polymyxin B, while the cytokine (IL-1β and IL-6) synthesis induced by *S. sanguis* LTA was blocked by 1.25μg/ml polymyxin B. IL-6 production in response to LPS was blocked by polymyxin B at 10μg/ml, but LPS-induced TNFα was significantly, though not completely, blocked, at polymyxin B concentrations greater than 10μg/ml.

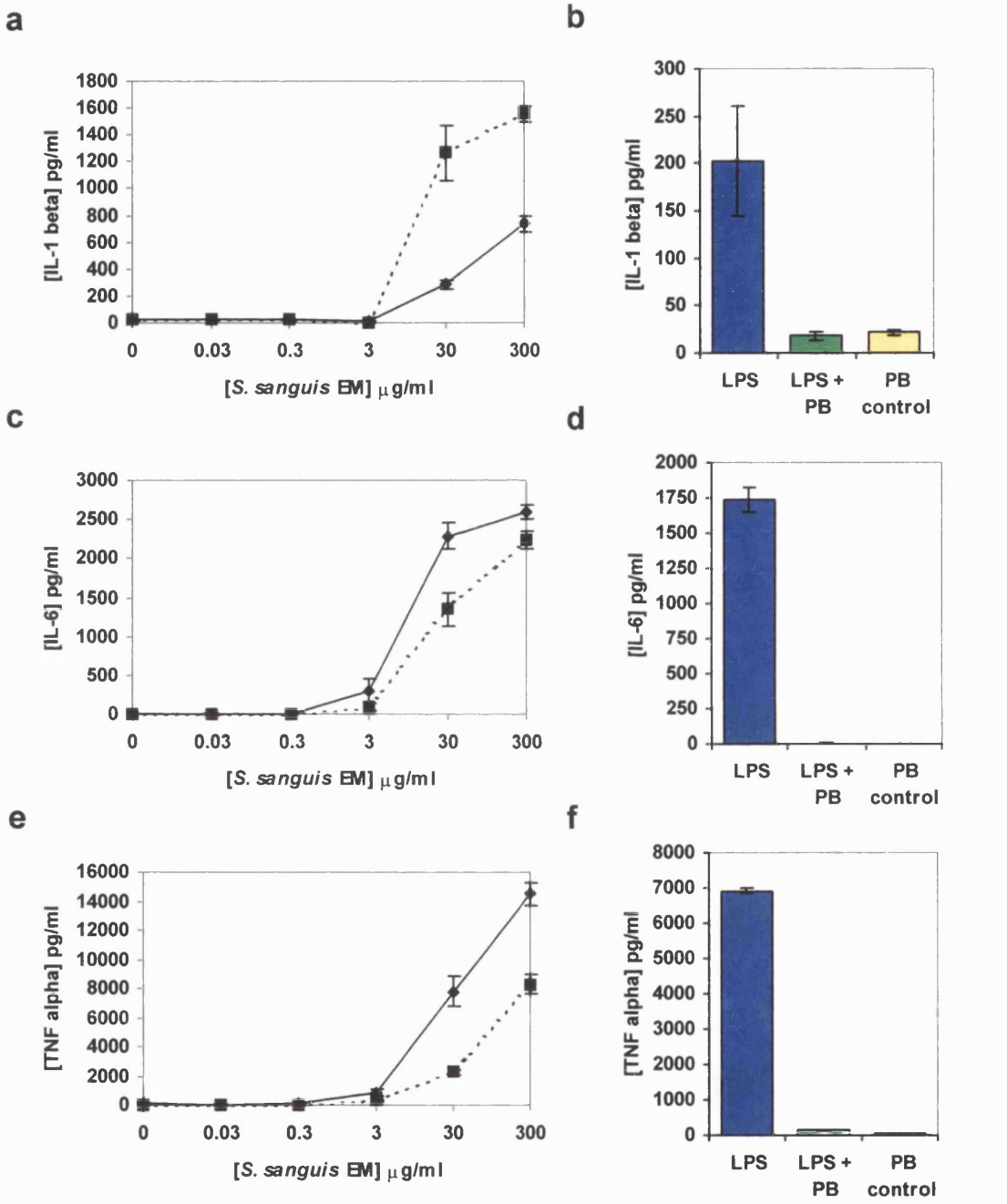

Figure 4.6 Effect of 20μg/ml polymyxin B on the cytokine-inducing activity of *S. sanguis* exported material. All data shown are from a single representative PBMC preparation, and are expressed as the mean and SD of triplicate cultures. Panels (a), (c) and (e) show the level of IL-1β, IL-6 and TNFα respectively, secreted from PBMC in response to various concentrations of *S. sanguis* EM tested in the absence (solid line) and presence (dashed line) of 20μg/ml polymyxin B. Panels (b), (d) and (f) show the level of IL-1β, IL-6 and TNFα respectively, secreted from PBMC in response to 10ng/ml *E. coli* LPS in the absence (blue bars) and presence (green bars) and of polymyxin B. The LPS served as a control to confirm the blocking activity of the polymyxin B.

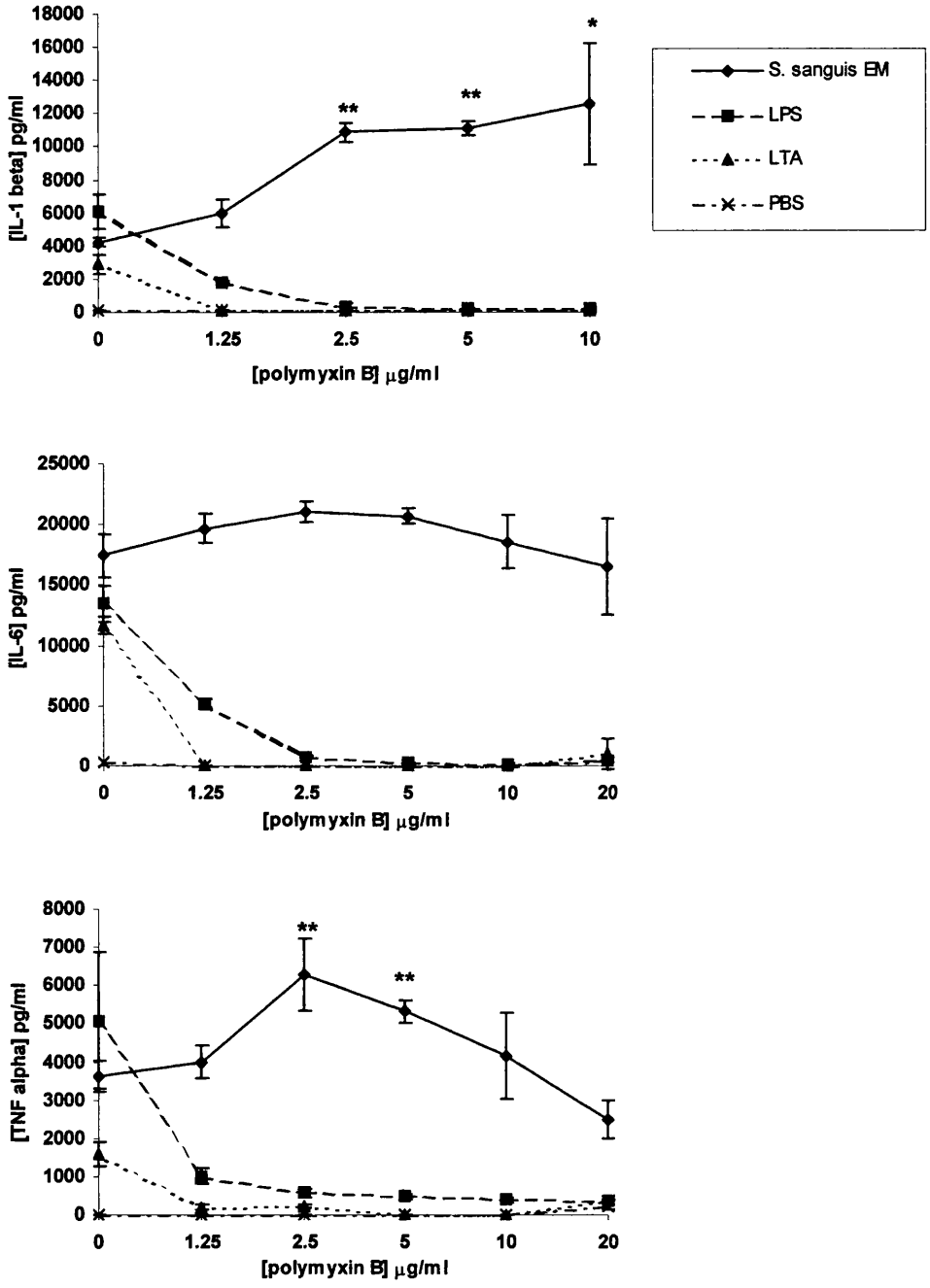

Figure 4.7 Effect of various concentrations of polymyxin B on the cytokine-inducing activity of *S. sanguis* EM, *S. sanguis* LTA and *E. coli* LPS. Results are expressed as the mean and SD of triplicate cultures. In each case, *S. sanguis* EM was used at 12µg/ml, LPS at 10ng/ml, and LTA at 100ng/ml, with equal volumes of PBS added as a negative control. Supernatants from cells tested in the presence of 20µg/ml polymyxin B were not tested for IL-1β. *$p<0.05$ and **$p<0.01$ when the amount of cytokine was compared to that induced in the absence of polymyxin B using a Dunnett's t-Test.

4.3.4.1 Effect of 20μg/ml polymyxin B on cytokine-induction by various concentrations of LPS and LTA

Polymyxin B at 20μg/ml was able to completely block IL-6 secretion from PBMC stimulated with up to 300ng/ml *E. coli* LPS, and up to 50μg/ml *S. sanguis* LTA (figure 4.8).

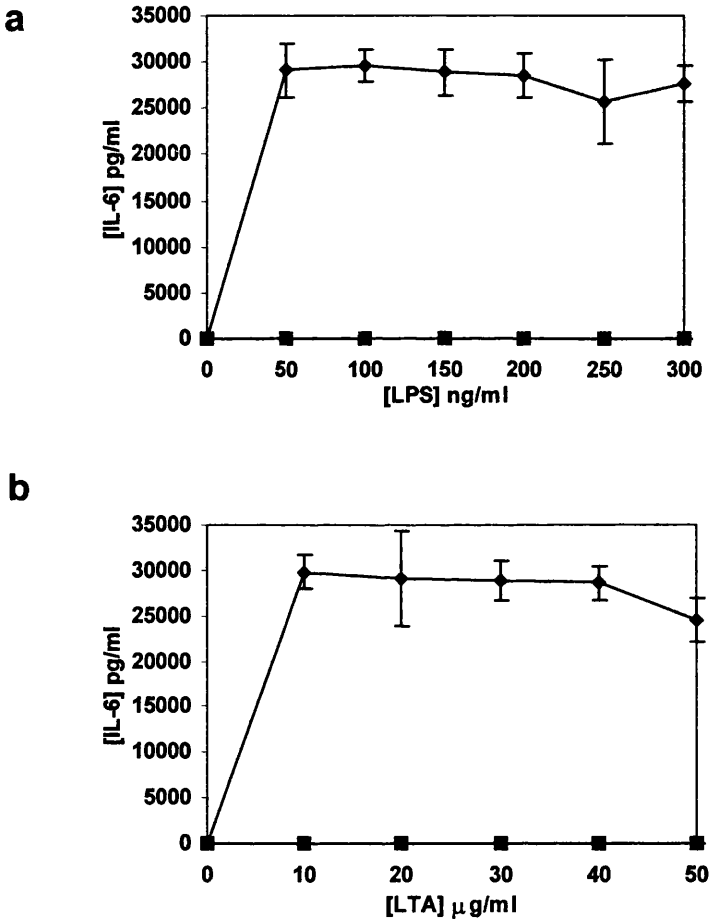

Figure 4.8 Effect of 20μg/ml polymyxin B on the IL-6-inducing activity of various concentrations of *E. coli* LPS and *S. sanguis* LTA. Results are expressed as the mean and SD of triplicate samples. Graphs show LPS-induced (panel (a)), and LTA-induced (panel (b)) IL-6 production by PBMC in the presence (square symbols), and absence (diamond symbols) of 20μg/ml polymyxin B.

4.3.5 Blocking experiments with anti-CD14 monoclonal antibodies

4.3.5.1 Effect of hybridoma supernatant containing anti-CD14 antibodies on the cytokine-inducing activity of *S. sanguis* EM

Undiluted hybridoma supernatant containing 60bca antibody (which binds to CD14) blocked LPS-induced IL-1β, IL-6 and TNFα production from PBMCs (Figure 4.9). The cytokine-inducing activity of *S. sanguis* EM at 3μg/ml (in terms of IL-1β, IL-6 and TNFα) was completely blocked by the hybridoma supernatant. However, inhibition of IL-6-induction was overcome (albeit to a limited extent) at concentrations of EM greater than 3μg/ml. Inhibition of IL-1β and TNFα-induction was also overcome, but a higher concentration of EM (greater than 30μg/ml) was required to do so.

4.3.5.2 Effect of purified 60bca antibody on the cytokine-inducing activity of *S. sanguis* EM

The preliminary experiments to assess the effect of the anti-CD14 monoclonal antibody, 60bca, on the cytokine-inducing activity of *S. sanguis* EM used hybridoma supernatant. To establish that the blocking activity was due to the antibody, a series of experiments was carried out using various concentrations of affinity-purified 60bca antibody. Purified 60bca at 3.75 and 7.5μg/ml inhibited LPS-induced IL-6 secretion by more than 97%, and at 15μg/ml inhibition was 99% (Figure 4.10a). At 3.75 and 7.5μg/ml 60bca, EM-induced IL-6 production was decreased by 97 and 96% respectively (Figure 4.10b). This blocking effect was slightly reduced when 15μg/ml 60bca was tested, but this was not due to the antibody itself inducing IL-6 secretion, since the amount of IL-6 produced in response to this concentration of 60bca alone was below the detection threshold of the ELISA (12pg/ml;data not shown).

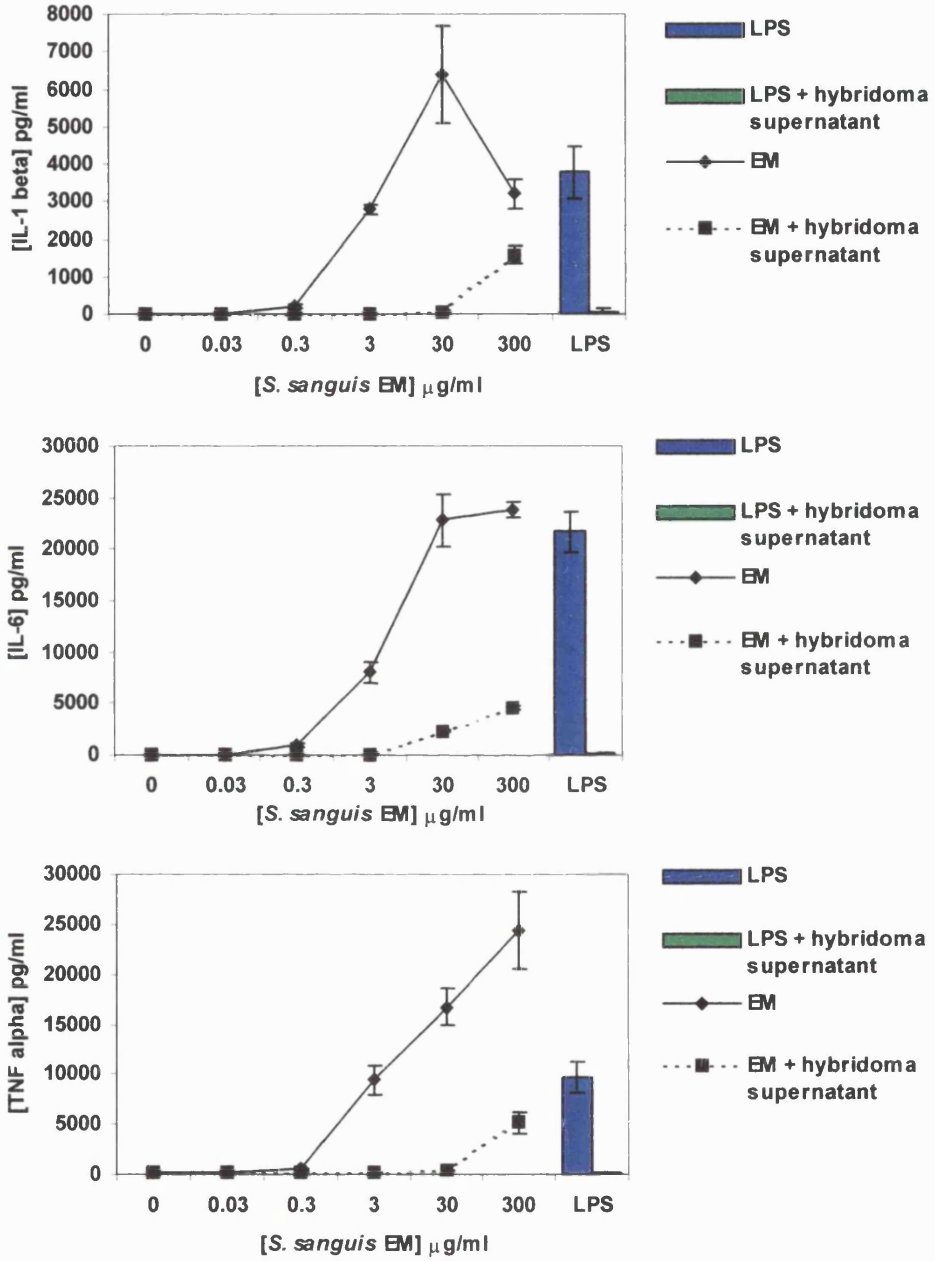

Figure 4.9 Effect of hybridoma supernatant containing anti-CD14 antibodies on the cytokine-inducing activity of *S. sanguis* exported material. All data are expressed as the mean and SD of triplicate cultures. Panels (a), (b) and (c) show the level of IL-1β, IL-6 and TNFα respectively, secreted from PBMC in response to various concentrations of *S. sanguis* EM tested in the presence (dashed line) and absence (solid line) of undiluted 60bca hybridoma supernatant. Also shown on each graph is the level of the relevant cytokine secreted from PBMC in response to 10ng/ml *E. coli* LPS in the absence (blue bars) and presence (green bars) of undiluted 60bca hybridoma supernatant. This served as a control to confirm the blocking activity of the hybridoma supernatant.

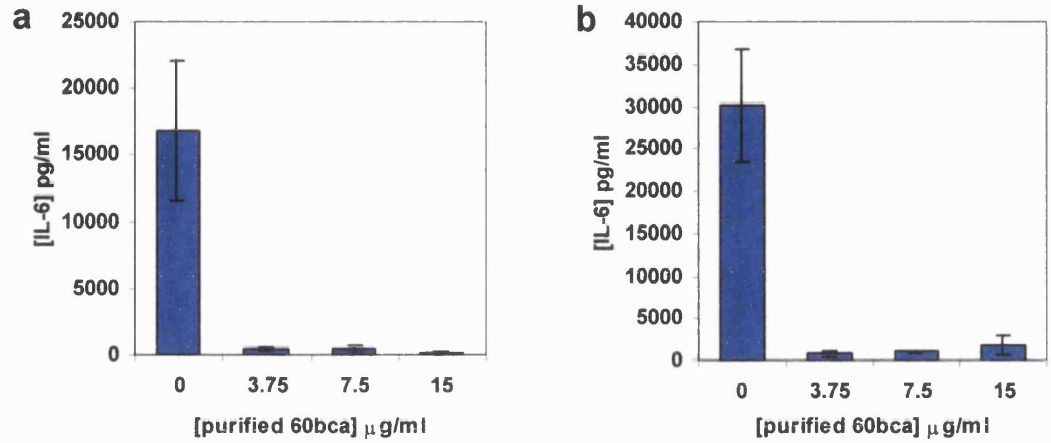

Figure 4.10 Effect of purified 60bca anti-CD14 antibodies on the IL-6-inducing activity of *E. coli* LPS and *S. sanguis* EM. Panel (a) shows *E. coli* LPS tested at 10ng/ml alone, and in the presence of 3.75, 7.5 and 15μg/ml purified 60bca antibodies. Panel (b) shows *S. sanguis* EM tested at 2μg/ml alone, and in the presence of 3.75, 7.5 and 15μg/ml purified 60bca antibodies. Results are expressed as the mean and SD of triplicate cultures.

4.3.5.3 Effect of 60bca and MY4 on the cytokine-inducing activity of *S. sanguis* EM

The IL-6-inducing activity of *E. coli* LPS, used as a positive control to determine the blocking activity of the anti-CD14 mAbs, was completely blocked by 60bca at 7.5μg/ml (Figure 4.11a). 60bca also inhibited the IL-6-inducing activity of *S. sanguis* EM in a dose-dependent manner: the amount of IL-6 secreted by PBMC was decreased by 69 and 83% when EM was tested in the presence of 7.5 and 15μg/ml 60bca respectively ($p<0.01$). The isotype-matched control for 60bca, a mouse IgG1 mAb, had no significant effect on the level of IL-6 secreted in response to *S. sanguis* EM. However, LPS-induced IL-6 secretion was increased by 33 and 34% in the presence of the control mAb at 7.5 and 15μg/ml ($p<0.05$). The control mAb itself did not induce significant IL-6 secretion from PBMC at either concentration tested.

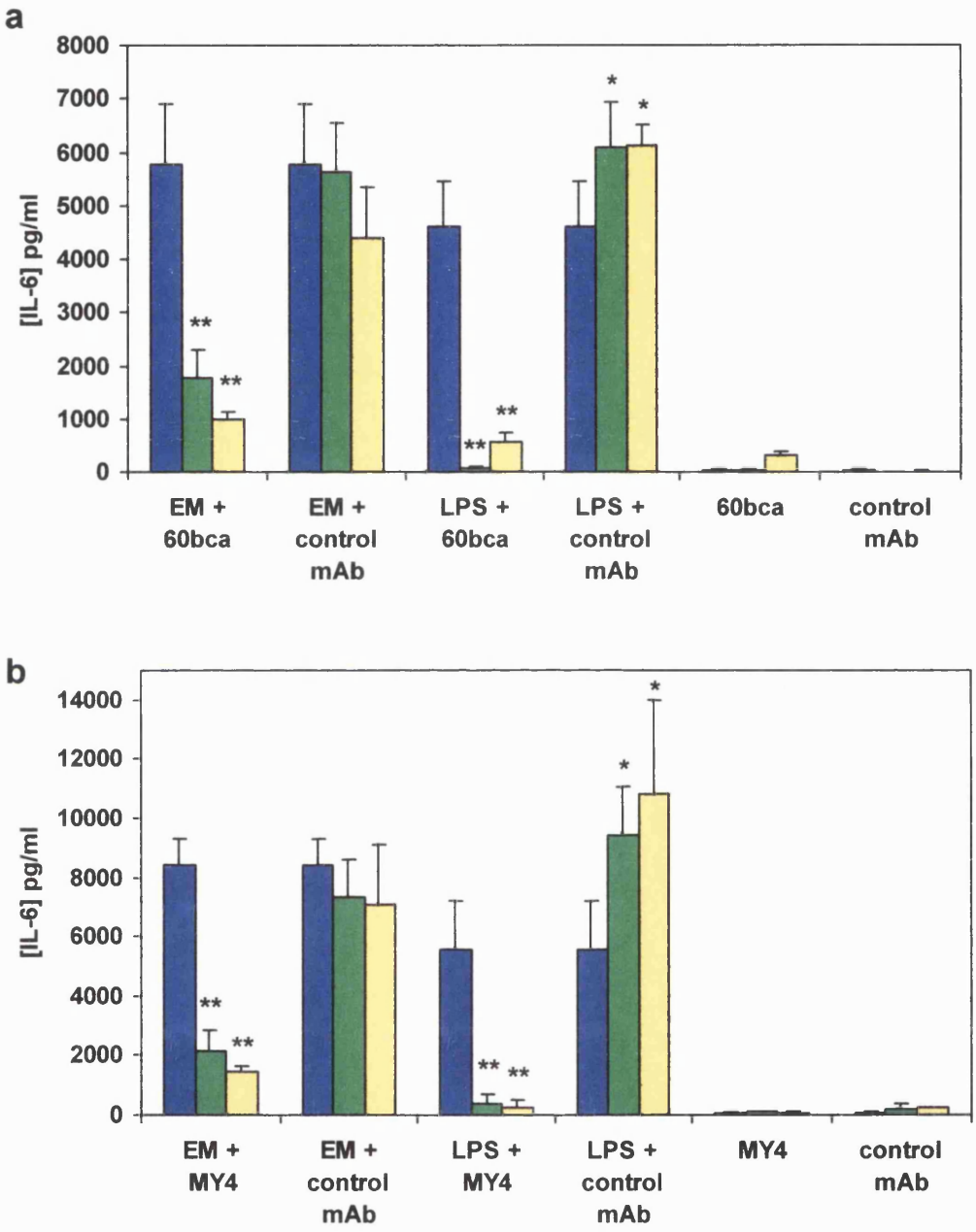

Figure 4.11 Effect of two different anti-CD14 monoclonal antibodies on the IL-6-inducing activity of *S. sanguis* EM and *E. coli* LPS. Panel (a) shows the effect of various concentrations of purified 60bca and its isotype-matched control on the activity of EM and LPS. Monoclonal antibodies were tested at 7.5μg/ml (green bars) and 15μg/ml (yellow bars). Panel (b) shows the effect of various concentrations of MY4 and it's isotype-matched control on the activity of EM and LPS. Monoclonal antibodies were tested at 5μg/ml (green bars) and 10μg/ml (yellow bars). Results are expressed as the mean and SD of triplicate cultures. *$p<0.05$ and **$p<0.01$ when LPS or EM tested in the presence of mAbs is compared to LPS or EM tested alone, using a Students t-Test.

The anti-CD14 mAb, MY4, decreased LPS-induced IL-6 secretion by 93 and 96% when tested at 5 and 10μg/ml respectively ($p<0.01$) (Figure 4.11b). *S. sanguis* EM-induced IL-6 secretion was also inhibited by MY4: IL-6 secretion was decreased by 74 and 83% by MY4 tested at 7.5 and 15μg/ml respectively ($p<0.01$). The isotype-matched control for MY4, a mouse IgG2b mAb, had no significant effect on EM-induced IL-6 production, but, as was observed for the IgG1 control mAb, LPS-induced IL-6 secretion was significantly enhanced by the presence of this mAb: IL-6 increased by 70 and 96% when LPS was tested in the presence of 5 and 10μg/ml respectively ($p<0.05$).

4.3.6 Time course of *S. sanguis* EM-induced cytokine production by PBMC

Figure 4.12 shows the production of IL-1β, IL-6, IL-8 and TNFα by PBMC after 0, 1, 2, 4 or 8 hours incubation with *S. sanguis* EM or *E. coli* LPS. There was no detectable production of IL-1β or IL-6 until 4 hours of incubation with either LPS or EM. In contrast, after just 1 hour of incubation with the same stimuli there was significantly more IL-8 and TNFα present than in the control supernatants from unstimulated PBMC ($p<0.01$ Students t-Test). By eight hours *S. sanguis* EM had induced a significantly higher level of both IL-1β and IL-6 secretion than LPS ($p<0.05$, Students t-Test) but there was no significant difference in the amount of IL-8 and TNFα induced by the two stimuli at the same time-point.

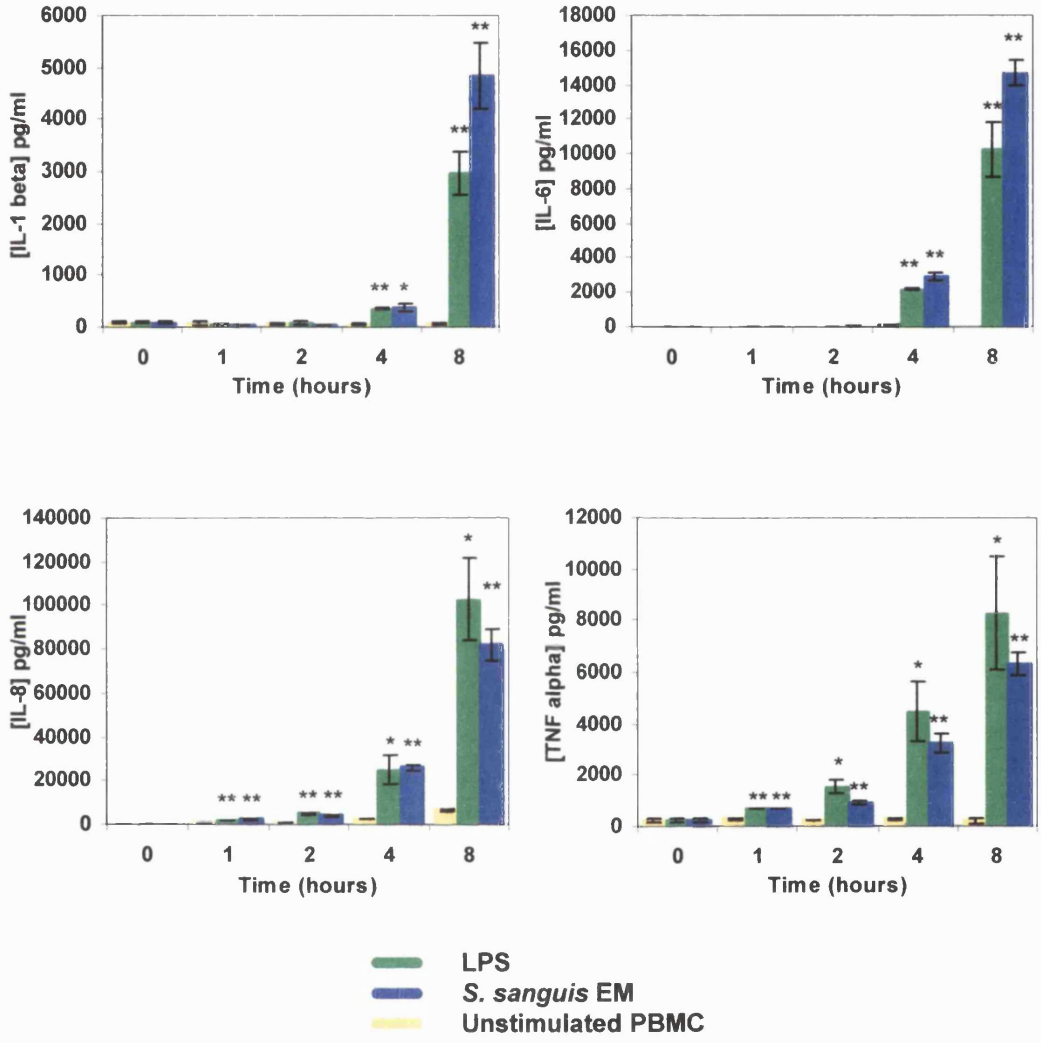

Figure 4.12 Cytokine production by PBMC stimulated with *S. sanguis* EM or LPS over an 8 hour time period. Results are expressed as the mean and SD of triplicate samples. *S. sanguis* EM was tested at 20μg/ml. *E. coli* LPS was tested at 10ng/ml. The amounts of IL-1β, IL-6, IL-8 and TNFα in culture supernatants were measured after 0, 1, 2, 4 and 8 hours incubation with stimuli. *p<0.05, and **p<0.01 when the amount of LPS- or EM-induced cytokine is compared to the amount of cytokine present in supernatants from the unstimulated PBMC at the same time point, using a Students t-Test.

These results did not give any clue as to when the peak secretion of each cytokine occurred, and so a second time-course experiment was carried out with time-points of 0, 4, 8, 20 and 24 hours. In response to LPS, the amount of IL-1β, IL-6 and IL-8 secreted by PBMC reached a plateau between 20 and 24 hours, a possible indication that no further cytokine secretion was occurring (Figure 4.13). For TNFα, the amount of cytokine appeared to plateau at some time between 8 and 20 hours. The peak level of each cytokine, produced in response to LPS, occurred at between the 8 and the 20 hour time points. The IL-1β and IL-6 responses to *S. sanguis* EM were similar, with both cytokines levelling off between 20 and 24 hours, while the TNFα response levelled off before the 20 hour time point. In contrast, the amount of IL-8 produced in response to the EM was still increasing significantly between 20 and 24 hours, while the IL-8 response to LPS had reached a plateau. Although the peak of IL-8 secretion could not be determined from these results, the other three cytokines appeared to have reached a maximal level at some time between the 8 and 20 hour time points.

4.3.7 Cytokine mRNA production by PBMC in response to *S. sanguis* EM over time

Figure 4.14 shows the RT-PCR products generated from of mRNA for IL-1β, IL-6, IL-8 and TNFα from PBMC which had been stimulated with *S. sanguis* EM for various lengths of time. Unstimulated PBMC were also sampled at the same time points. PCR using primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was carried out on all samples to demonstrate that similar amounts of cDNA had been used for analysis. A sub-maximal number of PCR cycles (25) was used to try and show differences between samples. Although there were strong signals for all samples tested for GAPDH, there were some differences in signal intensity both between different time-points, and between stimulated and unstimulated PBMC. Without the use of densitometry, the signals for the cytokines could not be normalised to take these differences into account. As a result, even though sub-maximal numbers of PCR cycles were used to try and give some idea of variations in the amounts of the cytokine

mRNAs, these results have been interpreted mainly in terms of the presence or absence of a particular mRNA.

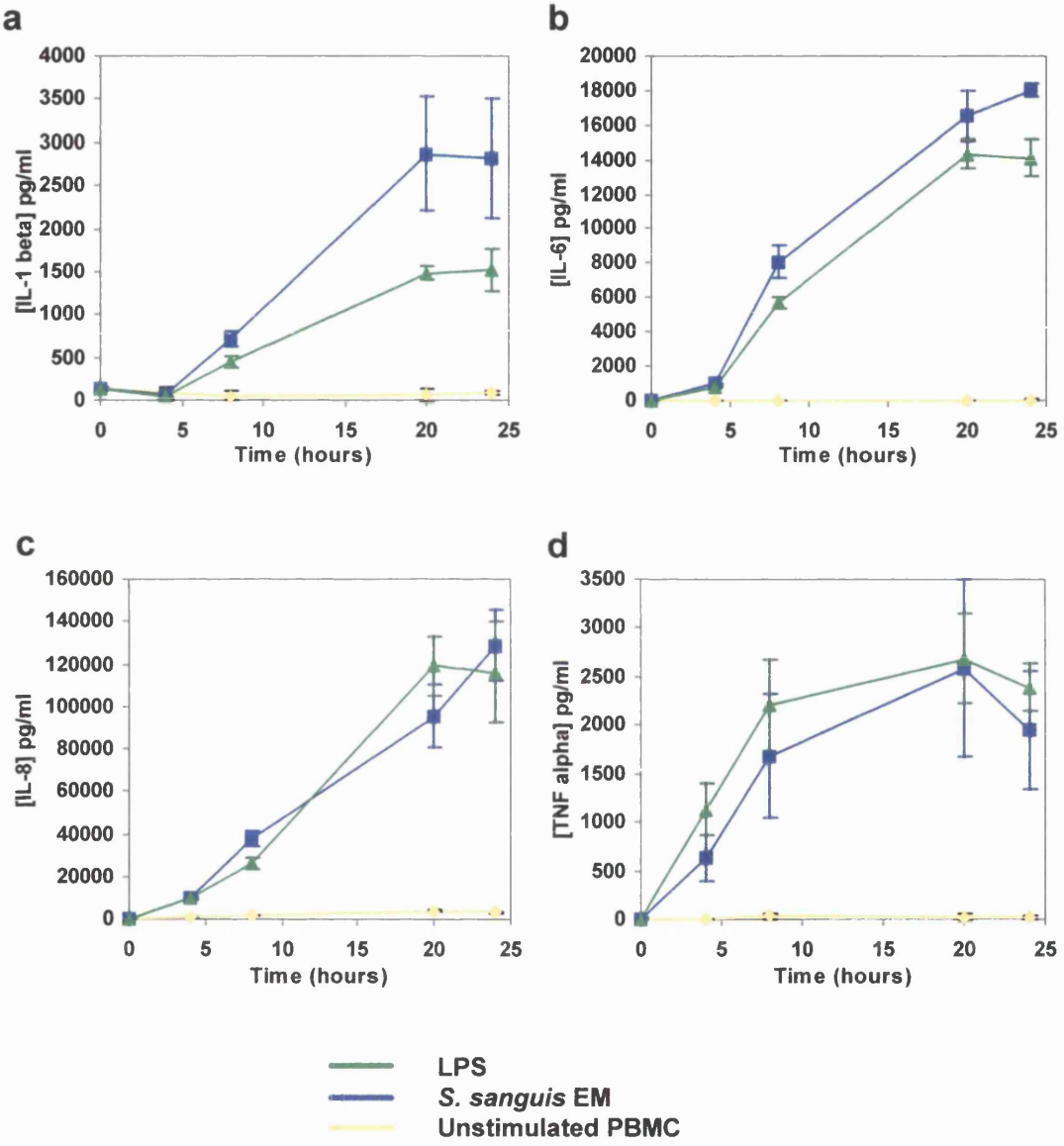

Figure 4.13 Cytokine production by PBMC stimulated with *S. sanguis* EM or LPS over 24 hours. Results are expressed as the mean and SD of triplicate samples. *S. sanguis* EM was tested at 20μg/ml. *E. coli* LPS was tested at 10ng/ml. The amounts of IL-1β (a), IL-6 (b), IL-8 (c) and TNFα (d) were measured in culture supernatants after 0, 4, 8, 20 and 24 hours incubation with stimuli.

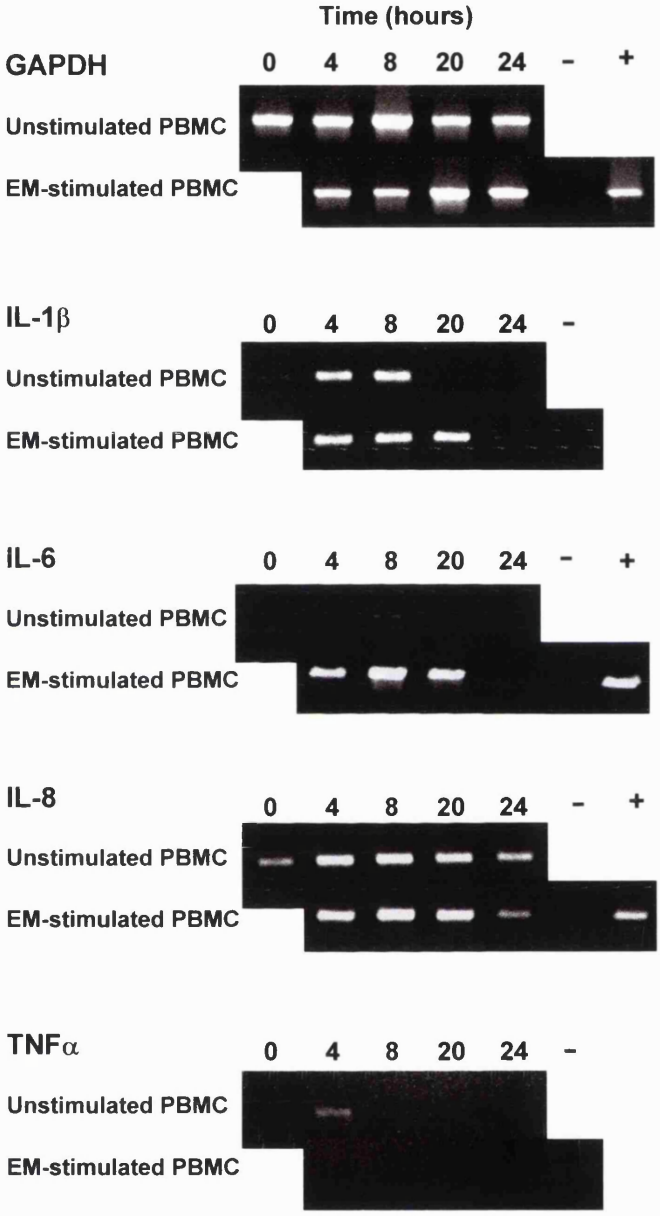

Figure 4.14 Effect of incubation with *S. sanguis* EM for various times on PBMC mRNA. PBMC were incubated with 20μg/ml *S. sanguis* EM for 0, 4, 8, 20 and 24 hours. RNA was extracted from cells (the cells used in the secretion of cytokines over time experiments (figure 4.13)) and used to generate cDNA. Amplification of signals was achieved using PCR with primers specific for GAPDH, IL-1β, IL-6, IL-8 and TNFα. Figures shown are photographs of ethidium bromide-stained gels. Lanes marked "-" and "+" represent controls included in the PCR reaction; these were H_2O (negative control), and a small amount of an appropriate PCR product from a previous experiment (positive control). Positive controls for IL-1β and TNFα were unavailable.

Messenger RNA for IL-1β was detectable by RT-PCR at 4, 8 and 20 hours, and only faintly at 24 hours, in cells stimulated with *S. sanguis* EM (figure 4.14). The apparent decrease in mRNA between 20 and 24 hours corresponded with the level of secreted protein, which did not increase further between 20 and 24 hours (Figure 4.13a). Signal was also detected in unstimulated cells at 4 and 8 hours, with a faint signal also detectable at 0 and 20 hours, but there was no corresponding secretion of IL-1β protein at any of the time-points tested (Figure 4.13a).

IL-6 mRNA was only just detectable in unstimulated PBMC at 4 and 8 hours (figure 4.14). In contrast, a strong signal was detected at 4, 8, and 20 hours in PBMC incubated with *S. sanguis* EM, although mRNA was no longer detectable after 24 hours. The level of secreted IL-6 protein continued to increase between 20 and 24 hours (Figure 4.13b), despite the disappearance of mRNA.

In cells incubated with *S. sanguis* EM there was detectable signal for IL-8 mRNA at all time points (figure 4.14) and a steady increase in secreted IL-8 (Figure 4.13c). In contrast, in unstimulated PBMC there was a detectable signal for IL-8 mRNA at all time-points sampled, including 0 hours, but no significant secretion of IL-8 (the detection limit of the ELISA was 14pg/ml).

The only sample showing detectable TNFα mRNA was from unstimulated PBMC at 4 hours (figure 4.14). No message was detectable at any of the time-points tested in cells stimulated with EM, despite the fact that the amount of TNFα secreted into the cell supernatant increased significantly between 4 and 8 hours (Figure 4.13d).

4.3.8 Effect of *S. sanguis* EM on cytokine mRNA transcription assessed using a cDNA array

Figure 4.15 shows the autoradiographs obtained by exposure of X-ray film to the hybridised arrays over seven days. The control array (Figure 4.15a) was hybridised with a probe generated from mRNA extracted from unstimulated PBMC, while the experimental array (Figure 4.15b) was hybridised with a probe prepared from mRNA extracted from EM-stimulated PBMC (8 hour stimulation). The hybridisation signals for most of the housekeeping genes in both the control and experimental array were of roughly equal intensity suggesting that the two probes had a similar incorporation of radiolabel. Hybridisation specificity was confirmed by the absence of signal for the negative control DNAs (plasmid and bacteriophage DNA; grid references 1J, 1K and 1L). Several cDNAs were considerably more abundant in the experimental probe than the control probe, as demonstrated by a substantially stronger hybridisation signal. In relation to the cytokines that have been investigated in other parts of this study, the levels of message for monocyte IL-1β and IL-8 were increased in EM-stimulated PBMC, while the signal for IL-6 mRNA was only slightly increased (figure 4.15 grid reference H3). No signal for TNFα was detectable on either array (grid reference I14). IL-17 was one of the most abundant cytokine mRNA species present in the stimulated PBMC, and was considerably more abundant than in unstimulated control cells. Messenger RNA for granulocyte colony stimulating factor (G-CSF) was also increased in EM-stimulated cells. Other cytokine/receptor mRNAs that had substantially stronger hybridisation signals than in the control cells are listed in Table 4.1. Message for chemokines other than IL-8, such as MIP-1α and MCP-1, did not appear to be upregulated in EM-stimulated cells at the time-point tested. Also of interest was the lack of upregulation of mRNA for any of the cytokines purported to inhibit inflammation, namely IL-1Ra, IL-4, IL-10, IL-13 and TGFβ.

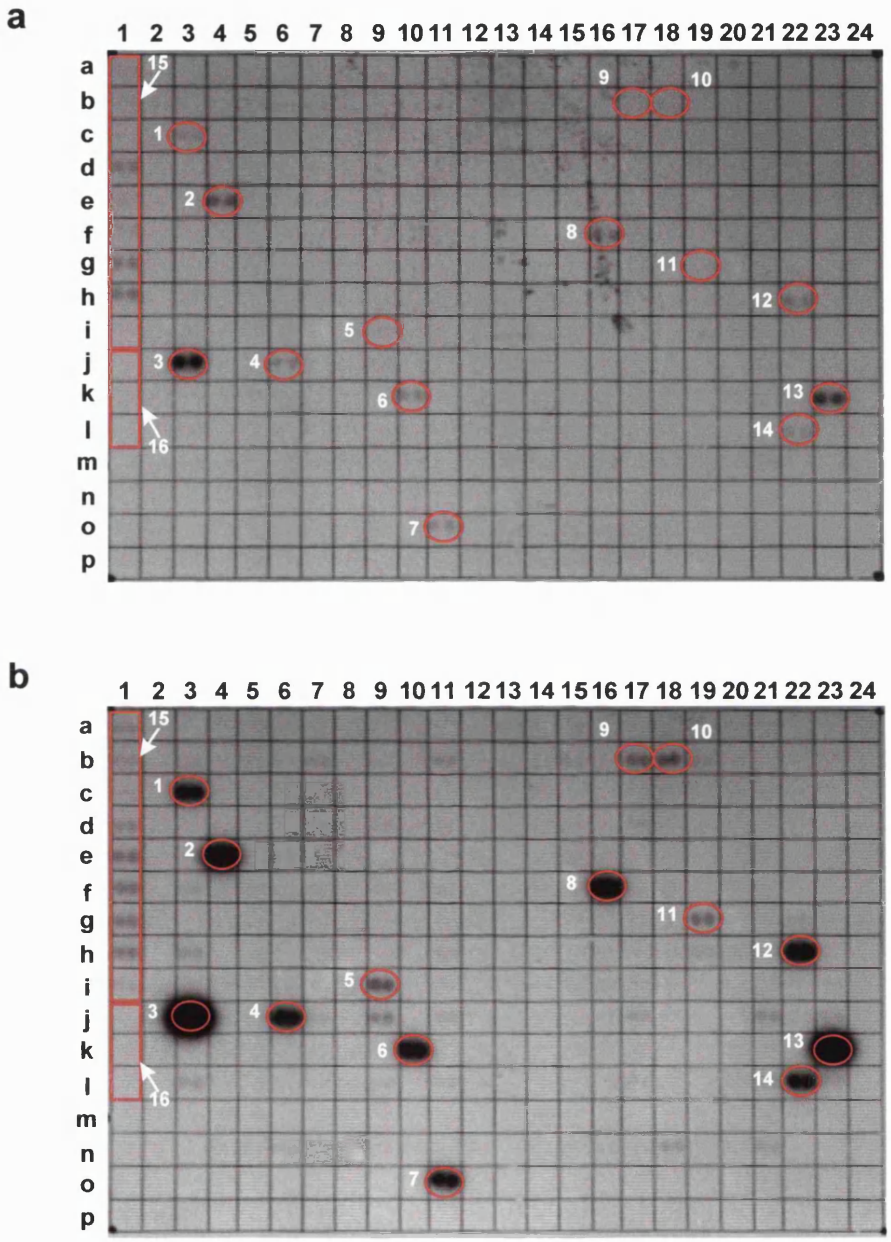

Figure 4.15 Analysis of the effect of *S. sanguis* EM on PBMC mRNA using a Clontech Atlas Human Cytokine/Receptor cDNA Array. Both panels show photographs of X-ray film exposed to the arrays for 1 week. Panel (a) shows the control array which was hybridised with a cDNA probe generated from RNA extracted from unstimulated PBMC. Panel (b) shows the experimental array which was hybridised with a cDNA probe generated from RNA extracted from EM-stimulated PBMC. Each grid square represents a different cDNA, spotted in duplicate onto the membrane. Messenger RNA for the following genes was present at a substantially higher level in the experimental cells: 1: IL-1β; 2: IL-8; 3: IL-17; 4: G-CSF; 5: cytokine receptor EBI-3; 6: LERK-3; 7: IGF-BP-3; 8: EDF; 9: FGF-8; 10: TDGF1; 11: CD135; 12: follistatin-related protein precursor; 13: receptor tyrosine kinase; 14: HM74; 15: housekeeping control cDNAs; 16: negative control DNAs.

Table 4.1. Cytokines/receptors that were upregulated in PBMC stimulated with *S. sanguis* EM.

Name of protein/gene (alternative names given in parentheses)	Function of protein/gene and references	Reference number for Figure 4.15
Cytokine receptor EBI-3	Forms heterodimers with the p35 subunit of IL-12 and has thus been suggested to be an immunomodulator (Devergne *et al.* 1996; Devergne *et al.* 1997)	5
Receptor tyrosine kinase ligand LERK-3 (EPLG3)	A GPI-linked ligand which can activate the Eek receptor (a member of the Eph family of receptors, the largest subgroup within the tyrosine protein kinase receptor family) (Park and Sanchez, 1997)	6
Growth hormone-dependent insulin-like growth factor-binding protein (IGF-BP-3)	Modulates the activities of IGF. The human form has been shown to affect DNA synthesis (DeMellow and Baxter, 1988) and the mouse form was reported to affect cell growth (Liu *et al.* 1992)	7
Erythroid differentiating protein (EDF)	Megakaryocyte differentiation (Fujimoto *et al.* 1991; Nishimura *et al.* 1991)	8
Fibroblast growth factor-8 (FGF-8)	Mainly thought to be involved in vertebrate development	9
Teratocarcinoma-derived growth factor 1 (TDGF1)	Epidermal growth factor-related protein.	10
CD135 antigen (FL cytokine receptor precursor)	Belongs to a family of receptor tyrosine kinases, other members of which include the receptor for macrophage colony stimulating factor (M-CSF) (Turner *et al.* 1996)	11
Follistatin-related protein precursor	Shares some sequence homology with follistatin (Zwijsen *et al.* 1994), which, as well as its role in the regulation of the female reproductive cycle, is thought to play a role in erythropoiesis (Shiozaki *et al.* 1992)	12
TKT	Tyrosine kinase receptor (Karn *et al.* 1993)	13
HM74	Member of the superfamily of G protein-coupled receptors, other members of which bind leukocyte chemoattractants, including IL-8, C5a, N-formyl peptides (FP), and platelet-activating factor (Nomura *et al.* 1993)	14

4.4 Discussion

Soluble exported material (EM) from *S. sanguis* was investigated for its ability to elicit an inflammatory cytokine response from human PBMCs. IL-1β, IL-6, IL-8 and TNFα were secreted in response to this material in a dose-dependent manner. Concentrations of EM as low as 30ng/ml induced an IL-8 response, while approximately ten-fold more (300ng/ml) was required to induce IL-1β, IL-6 and TNFα. In comparison with the activity of *E. coli* LPS, which was active at the picogram level, *S. sanguis* EM appeared to be a much less potent inflammatory agent, although the amounts of inflammatory cytokines produced in response to this material were certainly comparable to that of LPS, if not greater. Notably, IL-8 production continued to increase in response to between 30 and 300μg/ml EM, reaching approximately 500ng/ml IL-8 in the culture supernatant, while LPS (at 10ng/ml) only induced secretion of IL-8 to a level of 150ng/ml. Concentrations of LPS above 10ng/ml did not result in further increases in IL-8 secretion from PBMCs at the cell density used in these experiments (2×10^6/ml), and so it would appear that *S. sanguis* EM has a greater capacity for cytokine induction than *E. coli* LPS.

Returning briefly to the question of potency (a subject which will be addressed further in Chapter 8, the general discussion), it is perhaps more pertinent to compare EM from *S. sanguis* with components from other Gram-positive organisms, rather than those from Gram-negative bacteria. Many components of Gram-positive organisms have been reported to exhibit cytokine-inducing activity with widely varying potencies, and these have been termed "Modulins" in an extensive review by Henderson and colleagues (Henderson *et al.* 1996a). The lipoteichoic acids are surface glycolipids, which are considered to be the Gram-positive equivalent of LPS. Thus the cytokine-inducing activity of a commercial preparation of *S. sanguis* LTA was investigated. This was active in inducing IL-1β, IL-6 and TNFα from PBMC at as little as 10ng/ml, a potency somewhat greater than that observed for *S. sanguis* EM. Unfortunately, the possibility (although unlikely) that this activity was due to contamination of the preparation with LPS cannot be ruled out , since LTA is reactive in the LAL assay (an

assay used to measure the amount of LPS). However, LTAs from other bacteria have been reported to induce cytokine secretion from PBMC to a similar extent (Standiford *et al.* 1994).

The majority of the cytokine-inducing activity of the EM could be attributed to a proteinaceous component, since both heat-treatment and trypsin-digestion resulted in an almost complete inhibition of cytokine induction.

Experiments using the LPS-binding and inactivating antibiotic, polymyxin B, were carried out to confirm the absence of endotoxin contamination. Its mechanism of action involves direct interaction with LPS and the formation of a complex (Coyne and Fenwick, 1993), but the antibiotic must sequester LPS rather than just coating the LPS lamellar phase (opsonisation). The hydrophobic components of polymyxin B are inserted into the non-polar interior of the LPS lamellae (Thomas *et al.* 1999).

When low concentrations of *S. sanguis* EM were tested, IL-6 and TNFα secretion by PBMC was reduced (although not blocked) in the presence of polymyxin B, but this appeared to be overcome at higher concentrations of EM. This suggested that the inhibiting activity of the antibiotic was a function of the molar ratio of polymyxin B to EM. Secretion of IL-1β, on the other hand, was substantially increased in the presence of polymyxin B.

To confirm the extent to which polymyxin B could block the cytokine-inducing activity of *S. sanguis* EM, and to further investigate the augmentation of EM-induced IL-1β secretion, a second series of experiments was carried out, using increasing amounts of polymyxin B with a fixed, sub-maximal concentration of EM. A sub-maximal amount was used to ensure that even slight inhibition by polymyxin B would be seen as a substantial decrease in the level of cytokine secreted. Concentrations of LPS and LTA were chosen that induced the secretion of similar amounts of IL-6 from PBMC to those observed with 12μg/ml EM. Polymyxin B did not significantly decrease EM-induced IL-1β, IL-6 or TNFα production, even at 20μg/ml; the amounts of LPS and LTA that

could be blocked by 20μg/ml polymyxin B were shown to be at least 300ng/ml and 50μg/ml, respectively. In this respect, the active component(s) of EM differed from both LPS and LTA. The finding that polymyxin B could block LTA activity was in agreement with the findings of Renzi and colleagues (Renzi and Lee, 1995). In this study, polymyxin B dose-dependently inhibited *Staph. aureus* LTA-induced ICAM-1 (intracellular adhesin molecule-1) expression by HUVEC, and 10μg/ml polymyxin B was able to completely block the ICAM-inducing activity. The ability of polymyxin B to block the activity of LTA is not surprising, given the structural similarity of this molecule with LPS: both are anionic amphiphilic glycolipids (having both hydrophobic and hydrophilic domains), and therefore likely to bind polypeptides such as polymyxin B which are both cationic and possess hydrophobic regions. However, it has also been reported that polymyxin B has no effect on LTA activity (Keller *et al.* 1992), and the reasons for these different findings are unclear.

Both IL-1β and TNFα secretion in response to EM were significantly increased in the presence of some concentrations of polymyxin B. This increased release of cytokines could be due to a number of factors. EM may have contained a molecule that inhibited IL-1β induction, but that could be bound and inactivated by polymyxin B, thus allowing increased secretion of IL-1β. There may have been a synergystic effect between some component of EM and polymyxin B; polymyxin B alone did not induce IL-1β secretion at the concentrations tested. Monocyte and macrophage IL-1β is initially synthesised as an inactive pro-form that is proteolytically-processed by the IL-1β converting enzyme, ICE or caspase-1, to the mature form before secretion (Miller *et al.* 1993). The accumulation of intracellular pools of IL-1β in monocytes was demonstrated by Chin and Kostura (Chin and Kostura, 1993). This study showed that cells that had been pre-stimulated with levels of LPS too low to induce IL-1 secretion, could subsequently be induced (using a second, higher dose of LPS) to release mature IL-1β, despite the disruption of new protein synthesis at this second stage. Polymyxin B has been reported to induce cytokine synthesis in PBMC (Jaber *et al.* 1998). It is feasible that in the experiments described in this chapter the prior incubation of PBMC with polymyxin B

might have induced the accumulation (but not secretion) of an intracellular pool of IL-1β, and that subsequent stimulation with *S. sanguis* EM led to the release of this pool in addition to the IL-1β synthesised in response to the EM. Why EM might have led to the release of such an intracellular pool could be due to effects on PBMC membrane integrity, something which could be determined by dye-exclusion experiments, but was unfortunately not investigated at the time. The increased IL-1β secretion may instead have been a result of the increased TNFα secretion, since TNFα induces IL-1β (Dinarello *et al.* 1986).

Polymyxin B has been reported to induce the synthesis of TNFα in PBMC. The experiments documented by Jaber and colleagues measured total TNFα production by lysing the cells (Jaber *et al.* 1998), something that was not investigated in the study reported here. Unlike IL-1β, an intracellular pool has not been described for TNFα. However, TNFα is initially synthesised as a membrane-associated protein that is cleaved by a metalloproteinase of the ADAM family to yield the soluble form (Robache-Gallea *et al.* 1997). This membrane-associated pro-form could be considered as a releasable pool, and *S. sanguis* EM may affect the processing of polymyxin B-induced pro-TNFα to the mature form at a variety of stages, as well as itself inducing TNFα secretion.

Whatever the reason for the increased cytokine release, the absence of an increase in IL-6 or IL-8 release in the presence of polymyxin B suggests stimulatory mechanisms that are distinct from those responsible for IL-1β induction, and that presumably not all the IL-6 or IL-8 induction is via IL-1β.

Initial attempts to determine the mechanism of action of *S. sanguis* EM focused on CD14, a molecule reported to be involved in inflammatory responses to an increasing number of bacterial components (Zhang *et al.* 1993; Soell *et al.* 1995; Kusunoki and Wright, 1996; Weidemann *et al.* 1997; Sellati *et al.* 1998; van Furth *et al.* 1999). It has been described as a "pattern recognition receptor", in that it enables cells to respond to a diverse array of bacterial constituents (Pugin *et al.* 1994). Preliminary experiments

using hybridoma supernatant containing the anti-CD14 mAb, 60bca, revealed that at low levels of EM (3μg/ml), these antibodies completely blocked secretion of IL-6 by PBMC. This suggested a necessity for the CD14 receptor in the induction of cytokine secretion in response to *S. sanguis* EM. However, at a ten-fold higher concentration of EM (30μg/ml) this inhibition showed signs of being overcome, with hybridoma supernatant no longer completely blocking IL-6 induction. The EM might activate PBMC by two distinct mechanisms, one of which is independent of CD14: it is known that Gram-positive bacteria are capable of activating monocytes in both a CD14-dependent and -independent manner (Cauwels *et al.* 1997). EM is a mixture of molecules that may include cytokine-inducing proteins of low abundance that exert their effects by CD14-independent receptor pathways. Their low abundance might result in their effects only being observed at high concentrations of EM.

The blocking of cytokine-inducing activity was not due to other components present in the hybridoma supernatant as purified 60bca was also able to completely block EM-induced IL-6 secretion. Neither was blocking confined to this particular mAb: another anti-CD14 monoclonal, MY4, inhibited IL-6 production to a similar degree. Although the two anti-CD14 mAbs inhibited both LPS and *S. sanguis* EM activity, only LPS-induced IL-6 production was enhanced by the presence of the control mAbs (IgG1 and IgG2b). *S. sanguis* EM activity remained unaffected by the control antibodies. LPS (and the f-polysaccharide from *S. mutans*) has been reported to enhance the expression of Fc receptors for IgG (RFc-g) on the surface of human monocytes (Benabdelmoumene *et al.* 1991). This may explain the enhanced IL-6 secretion when LPS was tested in the presence of the control mAbs, in that enhanced Fc receptor expression might lead to increased receptor cross-linking and cell activation if control IgG binds to these receptors.

An alternative explanation for the effect of anti-CD14 antibodies on EM-induced cytokine production involves the Toll-like receptors (TLRs). The TLRs constitute the signal transducing component for CD14-dependent activation of monocytes by various bacterial molecules, including LPS, LTA and peptidoglycan (Means *et al.* 2000). It is

possible that the active component of EM activates monocytes by direct binding to one of the TLRs, rather than to CD14. This binding may be sterically hindered by the binding of anti-CD14 antibodies to the CD14 molecule which is in close proximity to the TLR.

The finding that CD14-blocking antibodies significantly reduced EM-induced cytokine production may have been taken to suggest contamination of the preparation with LPS or LTA, both of which have been demonstrated to activate cytokine release from monocytes via CD14 (Dentener *et al.* 1993; Cleveland *et al.* 1996; Hattor *et al.* 1997). However, EM-induced IL-6 production was not blocked by polymyxin B, but was significantly reduced by trypsin digestion and completely blocked by heating. Coupled with the result of the LAL assay, which estimated that LPS accounted for less than 0.00002% of the EM, these results strongly suggested that the cytokine-inducing activity of EM was not due to LPS or LTA contamination, and was more likely due to proteinaceous components.

To examine the kinetics of the cytokine response of PBMC to *S. sanguis* EM in more detail, a series of time-course experiments was carried out. Both secreted protein and mRNA for IL-1β, IL-6, IL-8 and TNFα were examined. IL-8 and TNFα were secreted earlier than IL-1β and IL-6, and since TNFα modulates the expression of IL-1β (Dinarello *et al.* 1986), and IL-1β induces IL-6 (Granowitz *et al.* 1992a), it would be interesting to investigate whether EM-induced IL-1β and IL-6 secretion occurred directly, or indirectly via TNFα. That IL-8 secretion started at an earlier time-point than IL-1β was interesting, since blocking experiments by Dinarello's group demonstrated that 50% of the IL-8 produced by monocytes in response to endotoxin was induced via IL-1 (Porat *et al.* 1992). If more time were available, similar blocking experiments could be carried out to investigate to what extent, if any, EM-induced IL-8 production by PBMC is IL-1-dependent. The IL-8 response to EM was also interesting in that the level of secretion was still increasing even 20 hours after induction, although the response to LPS (and the response to EM with respect to the other cytokines) had begun to plateau by this point. The fact that IL-1β, IL-6 and TNFα began to level off,

even though the stimulus had not been removed from the medium, suggests that either the stimulus was removed from the system, perhaps by internalisation of the active moiety - LPS, for example, is internalised by monocytes (Kang *et al.* 1990) and neutrophils (Luchi and Munford, 1993) - or that these cytokines were exhibiting feedback inhibition. For example, IL-1β has been shown to induce IL-6 secretion from monocytes (Tosato and Jones, 1990), and IL-6 is an inhibitor of the production of both IL-1β and TNF (Schindler *et al.* 1990). Another example of this is seen in the regulation of TNFα by IL-10 (Fiorentino *et al.* 1991; Wanidworanun and Strober, 1993) If feedback inhibition of these cytokines is occuring, then an explanation for the continued release of IL-8 might lie in the ability of *S. sanguis* EM to interfere with the feedback inhibition of this cytokine, an ability that could have important pathological implications for the host.

The RT-PCR time course experiments were primarily carried out to give some idea of a suitable time-point to use for the cDNA array experiments, but revealed some interesting phenomena with regards to the possible mechanisms of cytokine modulation by *S. sanguis* EM. Although the results showed only the levels of RT-PCR products, this may give an indication of the mRNA levels. Analysis of mRNA for IL-8 produced the most intriguing result. There appeared to be little difference between EM-stimulated PBMC and unstimulated control cells, despite the former cells secreting huge amounts of IL-8, while the latter secreted no detectable IL-8. This suggested that the control of IL-8 production by EM may not have been exerted at the level of mRNA transcription, but at some other stage, perhaps translation or post-translational modification of the protein (IL-8 is produced as a precursor protein). IL-18 is another cytokine in which unstimulated PBMCs constitutively produce mRNA but do not actually secrete the protein. IL-18, like IL-1, IL-8 and TNF, is produced as a precursor protein that must be processed to its active form, and it is suggested that control of IL-18 release may be at the level of processing (Puren *et al.* 1999).

In contrast to IL-8, there was an obvious increase in IL-6 mRNA in stimulated versus control PBMC at all time-points tested, suggesting upregulation of gene expression in

response to EM. The picture for IL-1β was less clear. Only at 20 hours was there a marked increase in IL-1β mRNA compared to control cells. As was observed for IL-8, control cells contained transcript but did not secrete detectable protein, leading to the conclusion that EM-induced IL-1β regulation occurred at a non-message level, perhaps by controlling the processing of this cytokine by caspase-1. Messenger RNA for TNFα was not detected by RT-PCR at any of the time-points tested, despite the time-dependent secretion of large amounts of cytokine. However, RT-PCR was able to detect the presence of mRNA for TNFα in cells stimulated with LPS (data not shown). It is possible that the transcripts were produced earlier than the first time-point sampled (4h), and the transcript degraded by the time this sample was taken. However, degradation precludes further translation, and protein secretion only began to plateau after 8 hours. In light of this, there must have been a considerable delay between translation and secretion of mature TNFα for there still to be secretion occurring at the same rate after translation had terminated. Alternatively TNFα mRNA may have been transcribed, translated, and broken down extremely rapidly, and therefore not detected. Another possibility which could explain these results is that TNFα was not in fact produced at all, and that the ELISA was detecting something other than TNFα.

It would be interesting to examine the levels of cytokine transcripts at earlier time-points, in particular to clarify the TNFα response to EM. If this was repeated, differences in levels of mRNA would be more accurately determined by Northern blotting as this is a semi-quantitative technique, avoiding the amplification step of PCR. This technique was used by Standiford and colleagues to investigate the dose- and time-dependent expression of IL-8 mRNA in peripheral blood monocytes stimulated with LTA from *S. pyogenes* and *Staph. aureus* (Standiford *et al.* 1994). In addition, transcription rates of specific mRNAs could be determined using nuclear run-off assays.

cDNA arrays are powerful tools in that a single hybridisation experiment can generate a gene expression profile for hundreds of genes at a time. Recently, Wang *et al* reported the use of a commercial cytokine array to investigate cytokine gene expression in human monocytes activated by *Staph. aureus* and peptidoglycan (Wang *et al.* 2000). In

the work described in this thesis, a commercial cDNA expression array was used to compare levels of cytokine/receptor mRNA in PBMC stimulated with *S. sanguis* EM to those in unstimulated PBMC. The arrays included the cDNA of 268 cytokine/cytokine receptor/cytokine-associated genes, as well as housekeeping genes and negative controls of plasmid and bacteriophage DNA. The level of non-specific hybridisation was low, as shown by the absence of signal for the negative control DNA, and no signal was observed for the genomic DNA controls even after a seven day exposure of X-ray film to the arrays, suggesting that the samples were free from genomic DNA. Signals from the housekeeping genes were surprisingly weak, even after a seven day exposure. This may have been due to the hybridisation probe having a low radioactive specific activity, a possibility since the ^{32}P used for labelling was more than a week old. It may also explain why only about 20 out of a possible 268 cytokine/receptor genes gave clearly visible hybridisation signals on either array, and so where a signal is absent this cannot necessarily be interpreted as absence of expression of mRNA. Of the cDNAs that did give a signal, only about 14 showed a signal of stronger intensity on the experimental array than on the control array. Several spots appeared to have a marginally stronger signal than on the control membrane, but the difference was not much greater than that seen between the housekeeping controls on the two arrays, and so these mRNAs were not considered to be more abundant.

The amount of mRNA for IL-1β and IL-8 was increased in EM-stimulated PBMC, a result which agreed with the ELISA measurements of secreted cytokines produced by PBMC. Increases in these cytokine transcripts were not observed in the RT-PCR time-course experiments (apart from one time-point for IL-1β). However, since PCR amplification was used, the presence of small quantities of message generates signal, and so the unstimulated control cells may have ended up with similar amounts of RT-PCR product to stimulated cells, despite starting out with lower levels of mRNA.

Messenger RNA for IL-17, a cytokine which is preferentially produced by CD4+ T cells (Kennedy *et al.* 1996), was present in substantially larger amounts in EM-stimulated cells. IL-17 is involved in the pro-inflammatory response by enhancing the expression

of the intracellular adhesion molecule-1 (ICAM-1) in human fibroblasts, and by stimulating epithelial, endothelial cells or fibroblasts to secrete IL-6, IL-8, G-CSF and prostaglandin E2 (Yao *et al.* 1995; Fossiez *et al.* 1996; Fossiez *et al.* 1998). IL-17 is thought to stimulate TNF and IL-1 production from macrophages (Jovanovic *et al.* 1998). IL-17 has recently been implicated in the tissue destruction of rheumatoid arthritis by inducing matrix metalloproteinase-1 (MMP-1) release (Chabaud *et al.* 2000).

There was also a marked increase in G-CSF mRNA in EM-stimulated cells. G-CSF is secreted by many cells types including monocytes, endothelial cells, and fibroblasts, and induction is thought to involve IL-1 (Zsebo *et al.* 1988; Wang *et al.* 1996), and IL-17 (Fossiez *et al.* 1996), cytokines already established to be up-regulated in response to *S. sanguis* EM. To determine whether the increase in G-CSF transcript was a result of direct stimulation by EM, or indirect stimulation via IL-1, use could be made of agents that block IL-1 activity, such as the IL-1 receptor antagonist protein (IL-1ra). As far as the biological activity of G-CSF is concerned, the receptor for G-CSF (CD114) is present on monocytes and all cells of the neutrophil/granulocyte lineage. G-CSF has been demonstrated to modulate IL-1 and TNFα release from monocytes (Boneberg *et al.* 2000). Haematopoetic progenitor cells committed to the neutrophil/granulocyte lineage are stimulated to proliferate and differentiate, while fully differentiated neutrophils are activated in response to this cytokine. In addition, G-CSF induces the synthesis of Formyl-Met-Leu-Phe (fMLP) receptors, which enhances the neutrophil response to bacteria secreting fMLP by prolonging the production of oxygen radicals (Avalos *et al.* 1990). Following on from this, mRNA for the receptor HM74 was increased in EM-stimulated PBMC. HM74 is a member of the superfamily of G protein-coupled receptors, other members of which bind leukocyte chemoattractants, including IL-8, C5a, fMLP, and platelet-activating factor (Nomura *et al.* 1993)

Transcript for the cytokine receptor EBI-3 was upregulated by EM. This receptor is secreted by B lymphocytes in response to Epstein Barr Virus infection. EBI-3 shares homology with the p40 subunit of IL-12 (the soluble receptor) (Devergne *et al.* 1996)

and can form heterodimers with the p35 subunit of IL-12 (the ligand) (Devergne *et al.* 1997). EBI-3 has thus been purported to be an immunomodulator, with potential to interfere with the functions of IL-12, a key cytokine in the differentiation of Th_0 precursor T cells to Th_1 cells. Broadly speaking, Th1 lymphocytes orchestrate phagocyte-dependent responses, as opposed to Th2 lymphocytes, which control phagocyte-independent immune responses (Henderson, 1998). An ability to inhibit the generation of a Th1 response by interfering with IL-12, could represent an advantage for an organism keen on avoiding phagocytosis, although in the case of *S. sanguis* growing within the relative safety of an endocardial vegetation, this may be less important.

Taken together, the upregulation of mRNAs for IL-1β, IL-8, IL-17, G-CSF, HM74 and EBI-3 points to an inflammatory immune response, with the emphasis on the recruitment of inflammatory cells, and although it must not be assumed that an increase in mRNA leads to an increase in secreted cytokine, other results have confirmed that IL-1β, IL-6, IL-8 and TNFα are all released by PBMC in response to *S. sanguis* EM. Interestingly, the pattern of cytokine gene expression seen in this experiment appeared to be more closely related to that obtained by Eckmann and colleagues who examined intestinal epithelial cells stimulated with *Salmonella dublin* (Eckmann *et al.* 2000), than the pattern obtained by Wang and colleagues who investigated monocytes treated with *Staph. aureus* and peptidoglycan (Wang *et al.* 2000). Both groups used the same commercial cytokine arrays as used in the studies described in this thesis. Taking the top 20 most highly expressed genes in the work on *Staph. aureus*, IL-8 and IL-1β were the only cytokine genes that were also significantly upregulated in the experiments described in this thesis. In contrast, *Salmonella dublin* induced the upregulation of IL-8, IL-17, LERK-3, G-CSF, HM74 and the Follistatin-related protein, all of which were significantly upregulated in response to *S. sanguis* EM.

In conclusion, the EM from blood-grown *S. sanguis* activates human PBMC to upregulate mRNA for a number of inflammatory cytokines, and to secrete IL-1β, IL-6, IL-8 and TNFα in a time- and dose-dependent manner. The active component(s) are most likely proteinaceous, and CD14 is sufficient (although not an absolute

requirement) to facilitate their effects. Chapter 5 documents the isolation of cytokine-inducing components from EM extracted from blood-grown *S. sanguis*.

CHAPTER 5

ISOLATION AND PURIFICATION OF CYTOKINE-INDUCING PROTEINS FROM THE EXPORTED MATERIAL OF BLOOD-GROWN *Streptococcus sanguis*

5.1 Introduction

Chapter 4 described the cytokine-inducing activities of the exported material (EM) from *S. sanguis* grown in the presence of blood. With the activity shown to be due to proteinacious elements, the investigation then turned to the isolation of the individual cytokine-modulating proteins using the ability to induce IL-6 release from PBMC as a screen for active proteins. The data reported in Chapter 3 showed the EM to contain a large number of as yet unidentified proteins, any of which might have been the active component, and so a variety of purification techniques were chosen in order to isolate and identify the cytokine-inducing protein or proteins. Given the blockade of cytokine-inducing activity by CD14 mAbs, it would have been sensible to try and isolate the active protein(s) on a CD14 column. Unfortunately the cost of making an affinity column of this sort was prohibitive, at least during this study.

The first choice of technique to isolate bioactive proteins was affinity chromatography. This may have seemed an odd choice of technique to start with in the purification of proteins about which little is known. However, knowledge of the molecule to be purified is not absolutely necessary with dye-affinity chromatography, since most proteins that bind to such matrices can be eluted with high salt concentrations, rather than by actual affinity elution. Dye-affinity chromatography has been used in numerous purification procedures (Lowe and Pearson, 1984; Scopes and Griffiths-Smith, 1984; Kroviarski *et al.* 1988; Jungblut and Klose, 1989; Regnault *et al.* 1992). In many cases the first step in the development of a purification procedure utilising dye-affinity chromatography involves screening a collection of dye ligands for the ability to bind to, and allow recovery of, the molecule of interest (Hey and Dean, 1983; Scopes, 1986). Since commercial kits containing a range of pre-packed dye columns were available, nine different ligands were screened for purification of proteins from *S. sanguis* EM.

Ion exchange chromatography is a widely used technique in protein purification. The principal interaction between the proteins and the ion exchanger occurs by electrostatic attraction, exploiting the different net charges on proteins at a given pH. It represents quite a versatile chromatography technique as the method of elution can be fine-tuned in various ways in order to optimise protein separation. In this investigation, anion exchange chromatography was chosen as at neutral pH most proteins are negatively-charged and thus bind to positively-charged matrices.

The final technique used in this part of the study was gel filtration chromatography, which allows the separation of proteins on the basis of their size. It is a gentler procedure than affinity or ion exchange chromatography since the proteins are not subjected to the stresses of moving between the liquid phase (dissolved) and the solid phase (adsorbed). Consequently, this method often gives almost 100% recovery of bioactivity, something of considerable importance in this study since the aim was to isolate active proteins.

5.2 Materials and Methods

5.2.1 Fractionation of *S. sanguis* EM using dye-affinity chromatography

The following buffers were prepared for use with a Dye Resin Test Kit:-

Equilibration buffer

10mM Tris-HCl pH 8.0 + 0.5mM $MgCl_2$

Elution buffer

10mM Tris-HCl pH 8.0 + 2.5M NaCl

Column regeneration buffers:-

1. 0.1M sodium borate pH 9.8 + 1M NaCl
2. 0.1M sodium borate pH 9.8

The dye resin kit (Sigma) consisted of 9 different columns with the following dye ligands covalently bound to agarose :-

R-2257 (reactive green 5)
R-2507 (reactive blue 4)
R-3757 (reactive yellow 3)
R-2382 (reactive yellow 86)
R-0503 (reactive red 120)
R-2882 (reactive green 19)
R-2757 (reactive brown 10)
C-1535 (cibacron blue 3GA)
R-2632 (reactive blue 72)

The columns (bed volume of 2.5ml) were washed through with 5 column volumes of distilled water to remove unbound dye, followed by 5 column volumes of equilibration buffer. Freeze-dried *S. sanguis* EM was dissolved in equilibration buffer to a concentration of 1mg/ml. 1ml was applied to each column and washed into the dye matrix by following immediately with 0.5ml equilibration buffer. Unbound protein was washed from the column with 5 column volumes of equilibration buffer and collected (total volume approximately 12ml). Bound protein was eluted with 5 column volumes of elution buffer, collected and stored at -20°C along with the unbound protein washes. Columns were regenerated with 10 column volumes each of regeneration buffers 1 and 2, followed by distilled water, and finally 2M NaCl.

5.2.1.1 SDS-PAGE analysis of fractions

Initial attempts to visualise either the unbound protein or eluted protein on SDS gels suggested that the samples were too dilute and in need of concentration prior to visualisation with silver stain. A small portion of each sample was therefore concentrated five-fold using Amicon Microcon 3 filtration units (3 kDa cut-off membranes; Millipore, UK). 40µl of each concentrated sample was separated on a 12% polyacrylamide gel and silver-stained, as described in Chapter 2, section 2.3. 20µg unpurified *S. sanguis* EM was also run on each gel for comparison of the banding pattern.

5.2.1.2 Assessment of IL-6-inducing activity of fractions in the routine PBMC assay

Various amounts of the concentrated unbound protein from the C-1535 and R-2757 columns were tested for IL-6-inducing activity in routine PBMC assays (Chapter 2,

section 2.5). The fractions were concentrated further using Amicon Microcon 3 units as before, and re-tested in the PBMC assay. After confirming by SDS-PAGE that the samples had not degraded, the PBMC assay was repeated using larger amounts of each fraction.

5.2.2 Fractionation of *S. sanguis* EM using an MA7Q anion exchange chromatography column

An MA7Q column was prepared for use on the Bio-Rad HRLC system as described in Chapter 2, section 2.8.3.1.3; buffer preparation is described in section 2.8.3.1.2. Details of the column volume, dimensions, buffers used etc., are given in section 2.8.3.1.1. 20mg of freeze-dried EM (from *S. sanguis* grown in the presence of blood) was dissolved in buffer A to a final concentration of 4mg/ml. The solubilised EM was dispensed into sterile 1.5ml Eppendorfs and centrifuged at 14,000 x g for 10 minutes at 4°C in a 5810R Eppendorf centrifuge with an FA-45-30-11 rotor, to remove undissolved particulates. Four fractionation runs were carried out in total, loading 1ml of the solubilised EM at a time. The separation was carried out at a flow-rate of 1ml/min with a salt gradient of 0-1M NaCl over 60ml. 1ml fractions were collected and the appropriate fractions from each run pooled, to give a total volume of 4ml for each fraction.

5.2.2.1 SDS-PAGE analysis of fractions

36µl of each fraction was separated on a 12% SDS polyacrylamide gel and silver-stained to reveal the protein bands, as described in Chapter 2, section 2.3.

5.2.2.2 Assessment of IL-6-inducing activity of fractions in the routine PBMC assay

Fractions were screened for IL-6-inducing activity using the routine PBMC assay as described in Chapter 2, section 2.5, with PBMC isolated from single donor buffy coat blood. 10µl of each fraction was tested in triplicate, and the resultant supernatants assayed for their IL-6 content by ELISA (Chapter 2, section 2.6)

5.2.3 Concentration and further separation of active fractions using a MiniQ anion exchange chromatography column

A MiniQ column was prepared for use on a SMART system as described in Chapter 2, section 2.8.3.1.3. Details of the column volume, dimensions and buffers used are given in Chapter 2, section 2.8.3.1.1. Fractions 17, 18, 19 and 20 from the previous fractionation were pooled and diluted 1 in 5 with buffer A to reduce the salt concentration and ensure efficient binding to the column. This resulted in a total volume of 80ml which was injected, 40ml at a time, onto the MiniQ column using a 50ml Superloop (Amersham Pharmacia Biotech, UK). During sample injection a maximum flow rate of 250µl/min was used to keep within the pressure limits of the Superloop; this was increased to 400µl/min during the actual elution method. The proteins were eluted with a 0-0.5M NaCl gradient over 20 column volumes (4.8ml) and the absorbance at 280nm was monitored. 100µl fractions were collected and stored at -20°C until needed.

5.2.3.1 Assessment of IL-6-inducing activity

Initially a 1 in 50 dilution of each fraction was prepared in sterile PBS and 10µl added in triplicate to cells in a routine PBMC assay (Chapter 2, section 2.5). This was equivalent to testing 0.2µl of each fraction. The level of IL-6 in the culture supernatants were measured by ELISA (Chapter 2, section 2.6). Since several of the fractions appeared to be highly active, the fractions were retested at a more dilute concentration to pinpoint the fraction with the greatest activity: 1 in 500 dilutions were prepared and 10µl of diluted fractions tested, equivalent to testing 0.02µl.

5.2.3.2 SDS-PAGE analysis

1µl of each fraction was separated on a 12% SDS polyacrylamide gel which was silver-stained to reveal the protein banding pattern (Chapter 2, section 2.3)

5.2.3.3 SDS-PAGE and electro-blotting of the active fraction for N-terminal sequencing

To assess whether there might be sufficient protein to allow N-terminal sequence analysis, 21µl of fraction 23 was reduced by heating with 4 x sample buffer (as described in Chapter 2, section 2.3.2), separated on a 12% gel and stained with Coomassie Blue (section 2.3). Since there appeared to be sufficient protein for sequencing, a second gel was run in the same way and electro-blotted onto PVDF membrane using the CAPS buffer system (Chapter 2, section 2.4.1.2) The membrane was stained with 0.1% Coomassie Blue (section 2.4.1.2.1). Both the 60 and the 23kDa protein bands were subject to N-terminal sequence analysis, as described in Chapter 2, section 2.4.1.2.2.

5.2.3.4 Assessment of the IL-6-inducing activity of a commercial preparation of a bacterial superoxide dismutase in the routine PBMC assay

Superoxide dismutase (SOD) from *Bacillus stearothermophilus* (Sigma) was dissolved in PBS to give a 1mg/ml solution. A series of ten-fold dilutions of this stock solution were prepared and various amounts of SOD added in triplicate to a routine PBMC assay as described in Chapter 2, section 2.5. The amount of IL-6 secreted by the PBMC in response to SOD was measured by ELISA (section 2.6). In some experiments SOD was tested in the presence of 10µg/ml polymyxin B, with LPS at 10ng/ml used as a control for the blocking effect of polymyxin B.

5.2.4 Concentration and further separation of fraction 15 using a MiniQ anion exchange chromatography column

Fraction 15 from the initial separation of *S. sanguis* EM by anion exchange chromatography using the MA7Q column (section 5.2.2) was separated using a MiniQ column as described in section 5.2.3. Briefly, the fraction was diluted 1 in 5 with buffer A (final volume 20ml) and injected onto the column using a 50ml Superloop. Proteins were eluted over a 0-0.5M NaCl gradient and 100µl fractions collected and stored at -

20°C until needed. 1µl of each fraction was separated on a 12% SDS polyacrylamide gel and silver-stained as described in Chapter 2, section 2.3. To assess the IL-6-inducing activity of the fractions, each was diluted 1 in 50 in PBS, and 10µl (the equivalent of 0.2µl) added in triplicate to cells in a routine PBMC assay, details of which are given in section 2.5. The level of IL-6 in the culture supernatants were measured by ELISA (Chapter 2, section 2.6).

5.2.4.1 Further separation of fraction 15 using a Superdex 75 gel filtration column

The most active fraction obtained from the separation of fraction 15 by anion exchange chromatography, fraction 25, (section 5.2.4) was further fractionated by gel filtration. A Superdex 75 gel filtration column was prepared for use on a SMART system as described in Chapter 2, section 2.8.3.2.3. Details of the column volume, dimensions and buffers used are given in section 2.8.3.2.2. Two fractionation runs were carried out, with 42µl of sample loaded each time. The fractionation was carried out at 40µl/min over a total of 3ml and 50µl fractions were collected. The elution of protein was monitored by absorbance at 280nm. Fractions were diluted 1 in 10 in PBS and 10µl added in triplicate to a routine PBMC assay to test for IL-6-inducing activity. 3µl of each fraction was separated on a 12% SDS polyacrylamide gel and silver stained.

5.2.4.2 SDS-PAGE of the active fraction

To determine the molecular mass of the protein in the active fraction, 3µl of fraction 7 was separated on a 6% SDS polyacrylamide gel and silver-stained as described in Chapter 2, section 2.4. As no band was visible on silver staining, the gel was repeated with 40µl of fraction 7, but this still revealed no protein band. In case the protein had stuck to the Eppendorf, 40µl of reducing sample buffer (Pierce & Warriner, UK) was added to the tube and heated in a boiling water-bath for 10 minutes. The whole sample was then separated on another 6% SDS polyacrylamide gel.

5.2.5 Repeat purification of the high molecular mass IL-6-inducing protein doublet

44mg of freeze-dried, blood-grown EM was dissolved in 11ml 20mM Tris pH 8.0 to give a 4mg/ml solution. This was then centrifuged to remove undissolved particulates as described in section 5.2.2. The solubilised EM was fractionated on a MA7Q column following the same method detailed in section 5.2.2, with a total of 11 runs carried out (1ml loaded per run). All fractions containing the high molecular mass protein as determined by analysis on 12% SDS polyacrylamide gels (fractions 10 to 17) were pooled (total volume 60ml) and concentrated to a final volume of 5ml using a 50ml ultrafiltration cell fitted with a YM10 membrane as described in Chapter 2, section 2.8.2. The sample was then diluted 1 in 5 with buffer A and injected onto a MiniQ anion exchange column using a 50ml Superloop. The fractionation method used was the same as that used previously (section 5.2.2) except that 50µl fractions were collected. At this stage, SDS-PAGE analysis on 12% gels showed that the high molecular mass protein was already well separated from the other proteins in the relevant fractions. It was therefore decided to proceed straight to electro-blotting, rather than adding in the extra purification step of gel filtration chromatography which would dilute the protein, as well as running the risk of loosing further protein.

5.2.5.1 SDS-PAGE and electro-blotting of the high molecular mass protein for N-terminal sequence analysis

32µl from each of five fractions containing the high molecular mass protein were heated with 4 x reducing sample buffer and separated on a 6% SDS polyacrylamide gel as described in Chapter 2, section 2.3. Proteins were blotted onto PVDF membrane using the CAPS buffer system (section 2.4.1.2) and stained with 0.1% Coomassie blue (section 2.4.1.2.1). The molecular mass of the high molecular mass protein was determined from the protein standards as described in Chapter 2, section 2.3.5. The membranes were sent to PE Applied Biosystems (UK) for N-terminal sequence analysis.

5.2.5.2 Western blot analysis of the 190kDa protein using antisera raised to P1 and CshA

On the basis of its size (190kDa), it was possible that the high molecular mass protein might have been a member of the P1 family of adhesins (which range from 180 to 210kDa (Soell *et al.* 1994)) expressed by several oral streptococci. This family includes the SspA and SspB adhesins for human salivary glycoprotein, expressed by *S. gordonii* (Demuth *et al.* 1996), (previously documented as the *S. sanguis* Ssp-5 adhesin (Demuth *et al.* 1990a; Demuth *et al.* 1990b), before changes in the classification deemed *S. gordonii* a distinct species). Both antiserum to the *S. mutans* SR antigen I/II-like protein (Moisset *et al.* 1994) and monoclonal antibodies to *S. mutans* P1 (Brady *et al.* 1991) have been shown to recognise *S. sanguis*. Since antiserum to the purified P1 (SpaP) antigen I/II protein of *S. mutans* serotype c was available (a kind gift from K. W. Knox, Institute of Dental Research, Sydney, Australia), it was decided to probe fractions containing the 190kDa protein with this serum. These antibodies are reactive with the *S. gordonii* antigen I/II polypeptides SspA and SspB (Demuth *et al.* 1996). Antiserum against another large adhesin, CshA from *S. gordonii*, was also tested. The antibodies were raised against a truncated 260kDa form of the CshA polypeptide (McNab *et al.* 1994), and were a kind gift from Dr R. McNab, Eastman Dental Institute.

5.2.5.2.1 Preparation of *S. sanguis* and *S. gordonii* mutanolysin digests

As positive controls for the detection of P1 adhesins by the antisera, mutanolysin digests of *S. sanguis* and *S. gordonii* were prepared as a source of P1-like polypeptides and CshA (McNab and Jenkinson, 1998). 10ml of overnight cultures of *S. sanguis* and *S. gordonii* (DL1-Challis) were grown up in Wilkins Chalgren broth. Bacteria were pelleted by centrifugation at 4000 x g for 10 minutes at 4°C in an Eppendorf 5403 centrifuge, and then washed twice with 1ml of sterile distilled water. Pellets were resuspended in 200µl of spheroplast buffer (20mM Tris-HCl, pH 6.8, containing 10mM $MgCl_2$, 26% (w/v) raffinose and 1mM PMSF). 20µl of mutanolysin (500 units; Sigma) was added to each sample and incubated for 1 hour at 37°C. Cells were pelleted by

centrifugation at 6000 x g for 10 minutes at 4°C. 150µl of supernatant was removed and mixed with 4 x reducing sample buffer before being separated by SDS-PAGE.

5.2.5.2.2 SDS-PAGE and Western blotting

20µl of each mutanolysin extract and 30µg *S. sanguis* EM were separated on two 8% SDS polyacrylamide gels as described in Chapter 2, section 2.3. One gel was silver-stained to check for the presence of the high molecular mass proteins. The other, which had duplicate lanes of each sample, was electro-blotted onto nitrocellulose membrane using the Tris-glycine buffer system (Chapter 2, section 2.4.1). After transfer of proteins, the lane containing the molecular mass markers was cut from the membrane and stained with Coomassie blue (section 2.4.2.1). The rest of the membrane was labelled, and each lane cut into individual strips. One strip from each sample was probed with either P1 or CshA antiserum. All incubations and washes were carried out on a rocking table. The membrane strips were first blocked for 1 hour in PBS containing 0.5% gelatin, before being incubated for 3 hours with the primary antibody diluted in PBS containing 0.1% gelatin (P1 antiserum was diluted 1 in 250; CshA antiserum was diluted 1 in 500). The strips were washed twice for 10 minutes in PBS 0.1% Tween 20, followed by a further two 10 minute washes in PBS. The secondary antibody, anti-rabbit-horse radish peroxidase (HRP) conjugate (Dako), was diluted 1 in 1000 in PBS containing 0.1% gelatin, and the membrane strips incubated in this for 1 hour. The membrane strips were washed as described above and developed with 3,3'-diaminobenzidine peroxidase substrate (DAB; Sigma) in accordance with the manufacturers instructions.

5.3 Results

5.3.1 Fractionation of *S. sanguis* EM using dye affinity chromatography

Figure 5.1 shows the SDS-PAGE analysis of the unbound protein from the dye affinity chromatography columns. A protein of approximately 56kDa was retained by column C-1535 (Figure 5.1a, lane 5), as indicated by its absence, and also to a small extent by column R-2257 (Figure 5.1b, lane 3). There were no bands visible on the gels run of

the eluted proteins, despite concentration of the samples. This suggested that the 56kDa protein had failed to elute from the columns. Although the 56kDa protein had not been recovered and so could not be tested for cytokine-inducing activity, it was decided to test the unbound protein from the C-1535 column to determine if the absence of this protein led to a reduction in activity. Unbound protein from column R-2757, to which the 56kDa protein did not bind, was also tested as a control. Unfortunately, even after several rounds of concentration, no activity was recovered in the samples, including the control. Due to the problems encountered with having to concentrate samples before they could even be visualised on SDS polyacrylamide gels, and the loss of activity, it was decided not to continue with this approach.

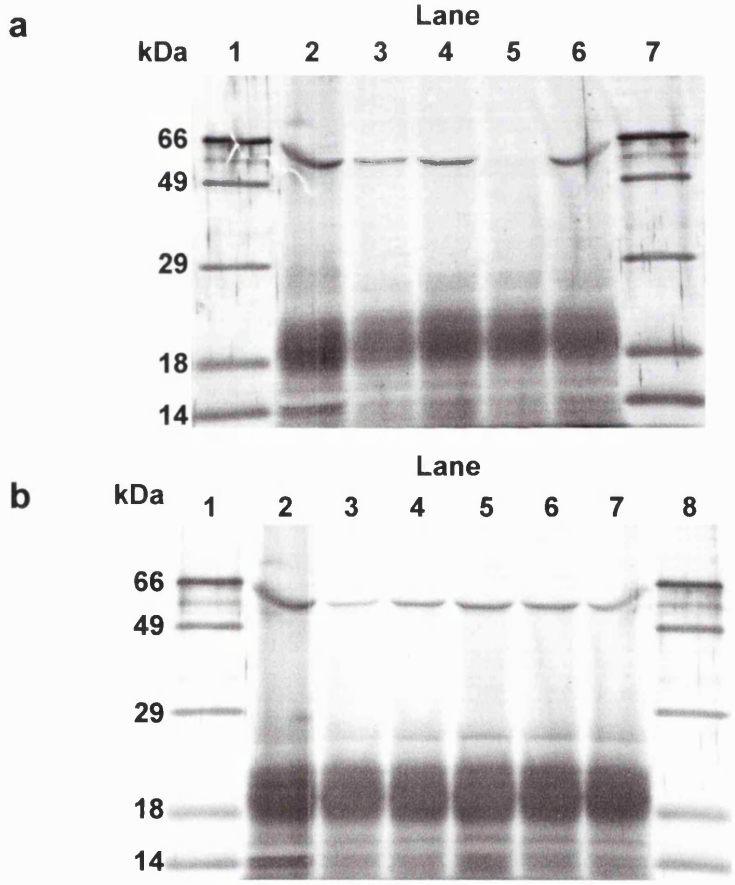

Figure 5.1 SDS-PAGE analysis of unbound protein from the fractionation of *S. sanguis* EM using a variety of dye affinity chromatography columns. 20µl of each sample was separated on a 12% SDS polyacrylamide gel and silver-stained. Panel (a) shows a photograph of gel 1. Lanes 1 & 7: molecular mass markers; Lane 2: *S. sanguis* EM; Lane 3: column R-2882; Lane 4: column R-2757; Lane 5: column C-1535; Lane 6: column R-2632. Panel (b) shows gel 2. Lanes 1 & 8: molecular mass markers; Lane 2: *S. sanguis* EM; Lane 3: column R-2257; Lane 4: column R-2507; Lane 5: column R-3757; Lane 6: column R-2382; Lane 7: column R-0503.

5.3.2 Fractionation of *S. sanguis* EM using anion exchange chromatography

To aid the reader, a flow diagram has been included so that the discussion of the purification schemes can be followed more easily (Figure 5.2).

Figure 5.3a shows the elution profile of *S. sanguis* EM fractionated on a MA7Q anion exchange chromatography column, with the amount of IL-6 released by PBMC stimulated with each fraction superimposed on the graph. The most active fractions were 19 and 20 (Active Peak 2), eluting at 0.23-0.25M NaCl, followed by fractions 15 and 16 (Active Peak 1), eluting at 0.16-0.18M NaCl. Concentrations of NaCl greater than 0.5M yielded two other less active peaks. Fractions 19 and 20 contained several proteins, as determined by SDS-PAGE, that could have been responsible for the activity in Peak 2, the most abundant of which was a protein of approximately 60kDa (Figure 5.3b). This protein was also present in large amounts in fractions 17 and 18, so it was decided to pool these along with 19 & 20 from Active Peak 2, concentrate them and attempt to further purify the active proteins. The description of the further purification of the proteins in Active Peak 1 is continued in section 5.3.5.

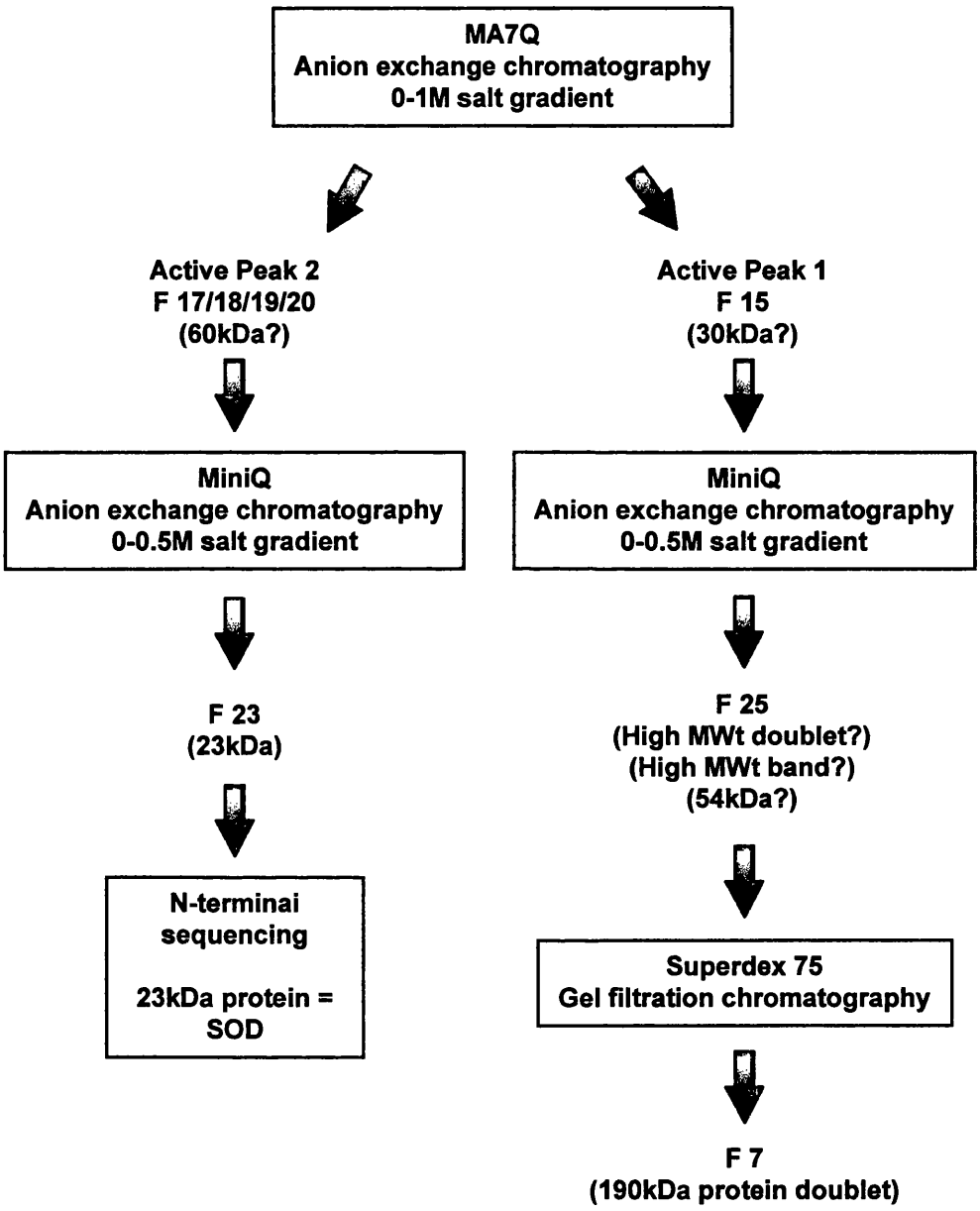

Figure 5.2 Flow diagram of the attempted isolation of IL-6-inducing proteins from *S. sanguis* EM. Purification steps and N-terminal sequencing are represented by rectangular boxes. Fractions are denoted by F followed by the fraction number. At each stage the molecular mass of the putative IL-6-inducing proteins is given in brackets beneath the appropriate fraction. The question mark indicates the protein(s) thought most likely to be the cytokine-inducing components at that stage in the purification.

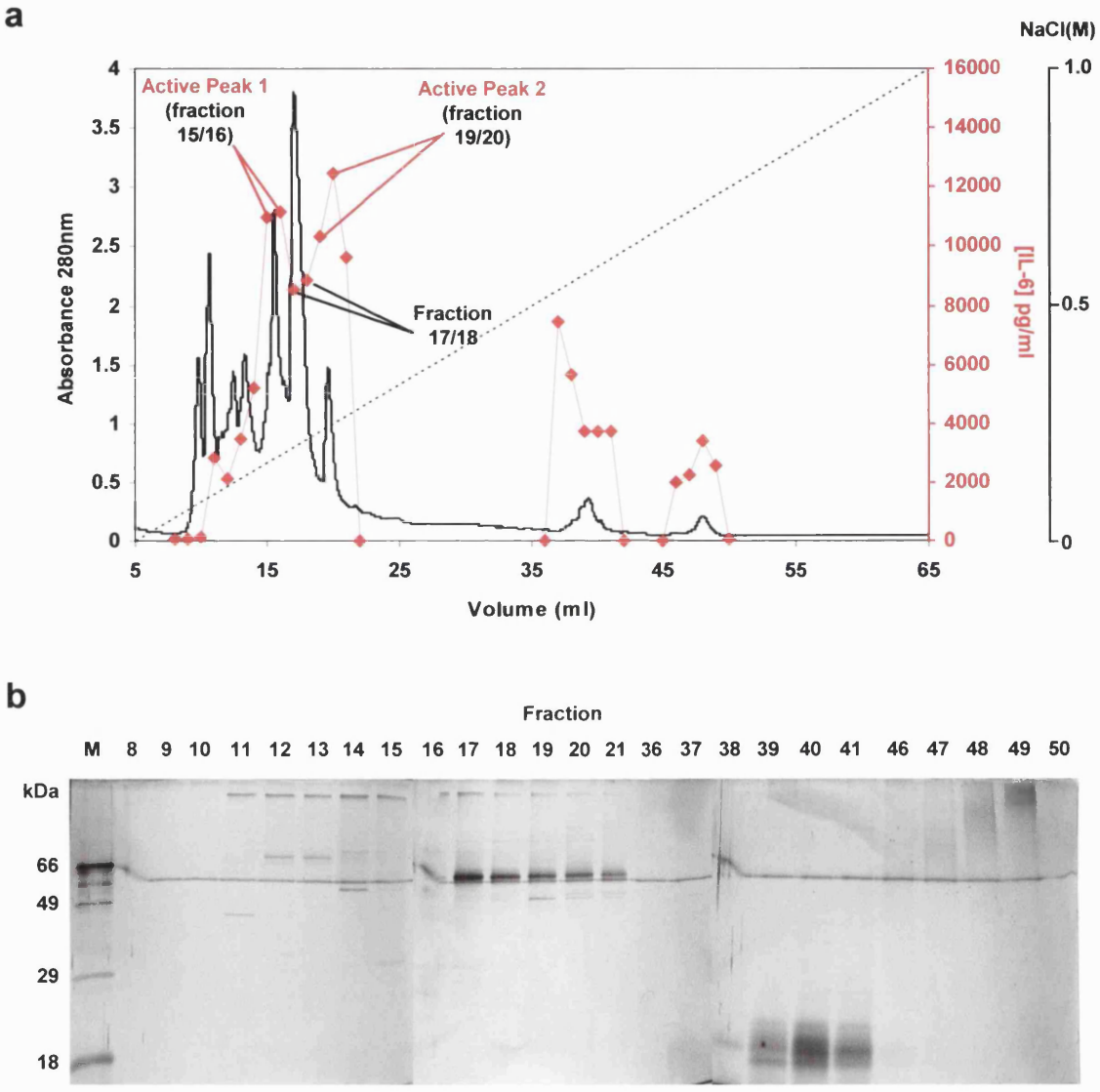

Figure 5.3 MA7Q anion exchange chromatography of blood-grown *S. sanguis* EM. Panel (a) shows the protein elution profile and the activity profile of the collected fractions. The elution profile is shown as absorbance at 280nm (black line), and the NaCl gradient (0-1M between 5 and 65ml) is shown by the black dotted line. 10μl of each fraction was assayed for IL-6-inducing activity (red line). Results are expressed as the mean of triplicate samples. Panel (b) shows the SDS-PAGE analysis of the collected fractions. 36μl of each fraction was separated on a 12% SDS polyacrylamide gel. The lane marked M shows the molecular mass markers. The number of each fraction corresponds to the volume during the fractionation at which it eluted. On a preliminary set of gels (not shown), fractions 22 to 35 and 42 to 45 contained no protein bands visible with silver stain and are therefore not shown here.

5.3.3 Concentration and further separation of the proteins in Active Peak 2 using anion exchange chromatography

Fractions 17,18,19 and 20 were pooled and re-fractionated using a MiniQ anion exchange chromatography column with a shallower salt elution gradient (0-0.5M as opposed to 0-1M NaCl). In addition to further separating the different proteins present in these fractions, this step also served to concentrate the proteins, since all the protein present in 16ml (the total volume of the four fractions) bound to the column and was subsequently eluted in a much smaller volume (approximately five fractions of 100µl each). Fractions were initially diluted 1 in 50 to test for IL-6-inducing activity, and this showed the activity to be centred on fraction 23, with slightly less activity in fractions 22 and 24 (Figure 5.4a, dotted line). This did not correspond to the 60kDa protein which stained most strongly in fraction 22, with the band gradually becoming less intense through fractions 23 to 25 (Figure 5.4b). The assay was repeated with ten-fold less of each fraction tested for IL-6-inducing activity, and the results confirmed that the activity did actually peak in fraction 23 (Figure 5.4a, solid line) and so was not due to the 60kDa protein. There was, however, a protein of approximately 23kDa that appeared to have eluted predominantly in fraction 23 (Figures 5.4b and 5.6). Figure 5.5 shows the elution profile of the MiniQ fractionation with the IL-6-inducing activity of the fractions (tested at a dilution of 1 in 500) superimposed.

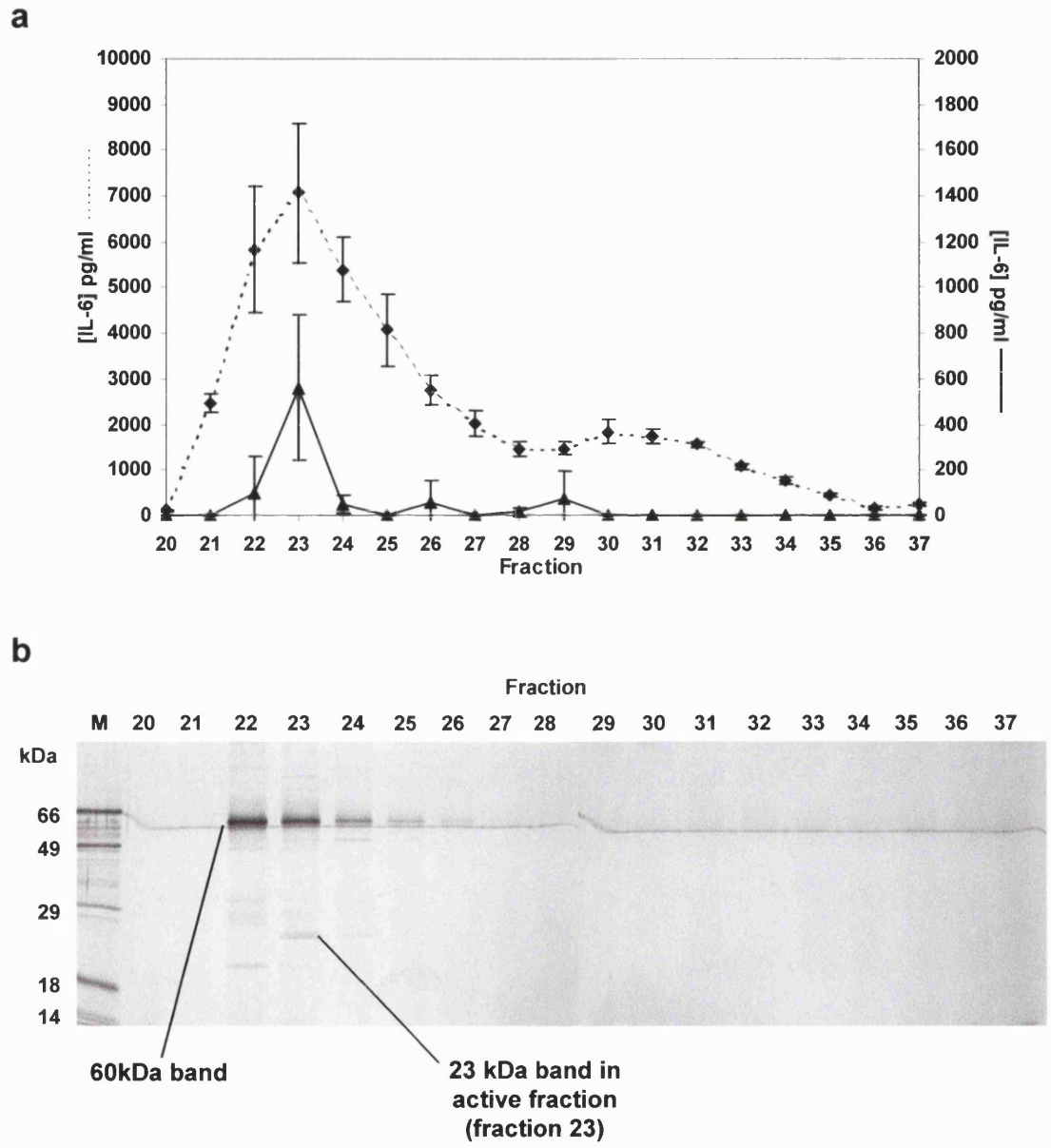

Figure 5.4 Concentration and further separation of Active Peak 2 (fractions 17, 18, 19 & 20) using a MiniQ anion exchange column. Panel (a) shows the IL-6-inducing activity of various dilutions of the fractions: the dotted line represents a 1 in 50 dilution (equivalent to the addition of 0.2μl) and is plotted against the left-hand axis, and the solid line represents a 1 in 500 dilution (equivalent to the addition of 0.02μl) which is plotted on the right-hand axis. Panel (b) shows the SDS-PAGE analysis of the fractions. 1μl of each fraction was separated on a 12% SDS polyacrylamide gel and silver-stained.

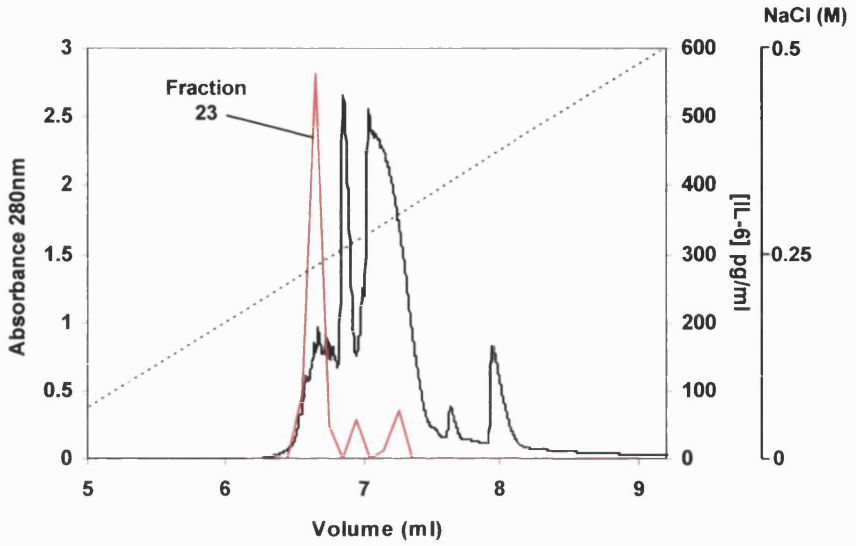

Figure 5.5 Concentration and further separation of Active Fraction 2 (fractions 17,18,19 & 20) using a MiniQ anion exchange column. The protein elution profile is shown as absorbance at 280nm (solid black line) and the elution gradient (0-0.5M NaCl between 4.4 and 9.2ml) is shown by the black dotted line. Fractions were assayed for IL-6-inducing activity and the data shown here is for fractions diluted 1 in 500 (equivalent of testing 0.02µl) (red line).

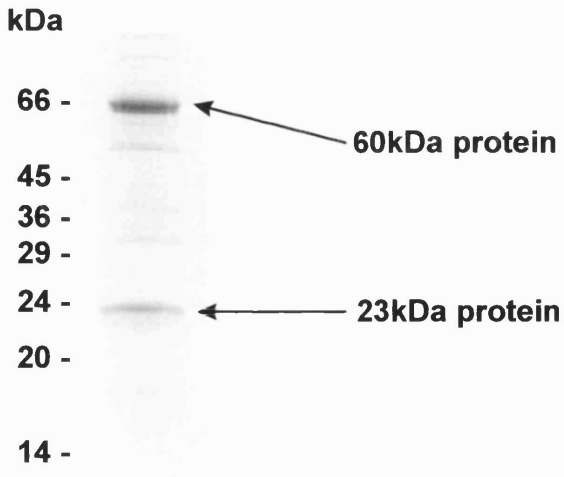

Figure 5.6 SDS-PAGE analysis of the active fraction, fraction 23 (17, 18, 19 & 20). The sample was separated on a 12% SDS gel and stained with Coomassie Blue. Both the 60 and the 23kDa proteins were N-terminal sequenced. No sequence data was obtained for the 60kDa protein, but the sequence obtained for the 23kDa protein shared 88% homology with that of the manganese-dependent superoxide dismutase (SOD) from *S. pneumoniae* and *S. mutans*.

5.3.3.1 N-terminal sequence analysis

No sequence data were obtained from the 60kDa protein. However, N-terminal sequencing of the 23kDa protein produced a continuous sequence of 18 amino acid residues. Comparisons using the NCBI BLAST network service showed this sequence to have 88% homology (in terms of amino acid identities) with the manganese-dependent superoxide dismutase (SOD) protein of *S. mutans*, matching 16 out of the first 18 amino acids (Figure 5.7).

S. sanguis 23kDa protein	A I L L P D L P Y A Y D A N E P Y I
S. mutans SOD	A I I L P D L P Y A Y D A L E P Y I

Figure 5.7 N-terminal amino acid sequences of the 23kDa protein from *S. sanguis* and the *S. mutans* SOD protein. Identities in the amino acid sequences are shown as blocked text. The third amino acid represents a conserved substitution.

5.3.4 Assessment of the IL-6-inducing activity of a commercial preparation of a bacterial superoxide dismutase

Superoxide dismutase from *B. stearothermophilus* induced PBMC to secrete IL-6 in a dose-dependent manner, with activity being seen at 0.7mg/ml SOD (0.02μM) (Figure 5.8a). At 7μg/ml, SOD induced the same amount of IL-6 from PBMC as 10ng/ml LPS. However, when SOD was tested in the presence of 10μg/ml polymyxin B, a concentration that blocked the activity of 10ng/ml LPS, the IL-6-inducing activity was completely blocked (Figure 5.8b). This suggested that the activity of the commercial preparation of SOD may not have been due to the protein itself, but to contaminating LPS.

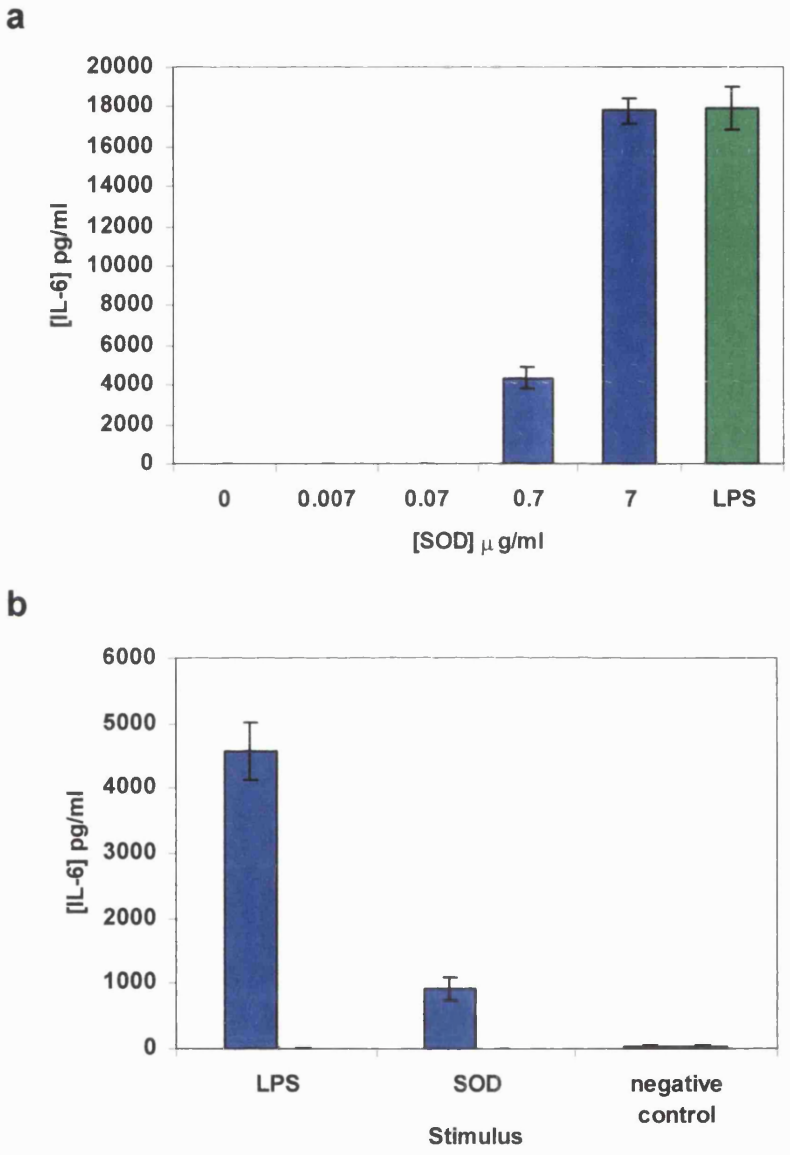

Figure 5.8 IL-6-inducing activity of a commercial preparation of SOD from *B. stearothermophilus*. All results are expressed as the mean and SD of triplicate cultures. Panel (a) shows IL-6 release from PBMC in response to various concentrations of SOD (blue bars). LPS at 10ng/ml was included as a positive control (green bar). Panel (b) shows the effect of polymyxin B on the IL-6-inducing activity of SOD. SOD was tested at 0.7μg/ml either alone (blue bar) or in the presence of 10μg/ml polymyxin B (green bar). As a control for the blocking effect of polymyxin B, LPS was tested at 10ng/ml either alone (blue bar) or in the presence of 10μg/ml polymyxin B (green bar).

5.3.5 Concentration and further separation of Active Peak 1 using a MiniQ anion exchange column

Of the fractions obtained from the initial MA7Q fractionation (section 5.3.2), fractions 15 and 16 were the next most active after fractions 19 and 20. Fractions 15 and 16 contained a protein of approximately 30kDa which appeared to be unique to these fractions and could be responsible for the IL-6-inducing activity (see Figure 5.3b). This band was extremely feint in fraction 16, so fraction 15 was chosen for further purification.

The proteins from fraction 15 were eluted from the MiniQ column using a shallower gradient than that used for the initial fractionation, and this resulted in the separation of the protein into several peaks (Figure 5.9a). The predominant peaks corresponded to fractions 25/26 and 35/36, and these are marked on the chromatogram. IL-6 inducing activity centred around fraction 25, corresponding to the first main peak of eluted protein (Figure 5.9b). None of the other fractions induced PBMC to secrete IL-6, including fractions 35 and 36. SDS-PAGE analysis revealed that the active fraction (fraction 25) contained three proteins visible with silver stain, the predominant being a high molecular mass protein which appeared to be a protein doublet (Figure 5.9b insert). Also present in fraction 25 were a single high molecular mass protein, and a protein of approximately 54kDa. None of the other fractions contained proteins visible with silver stain.

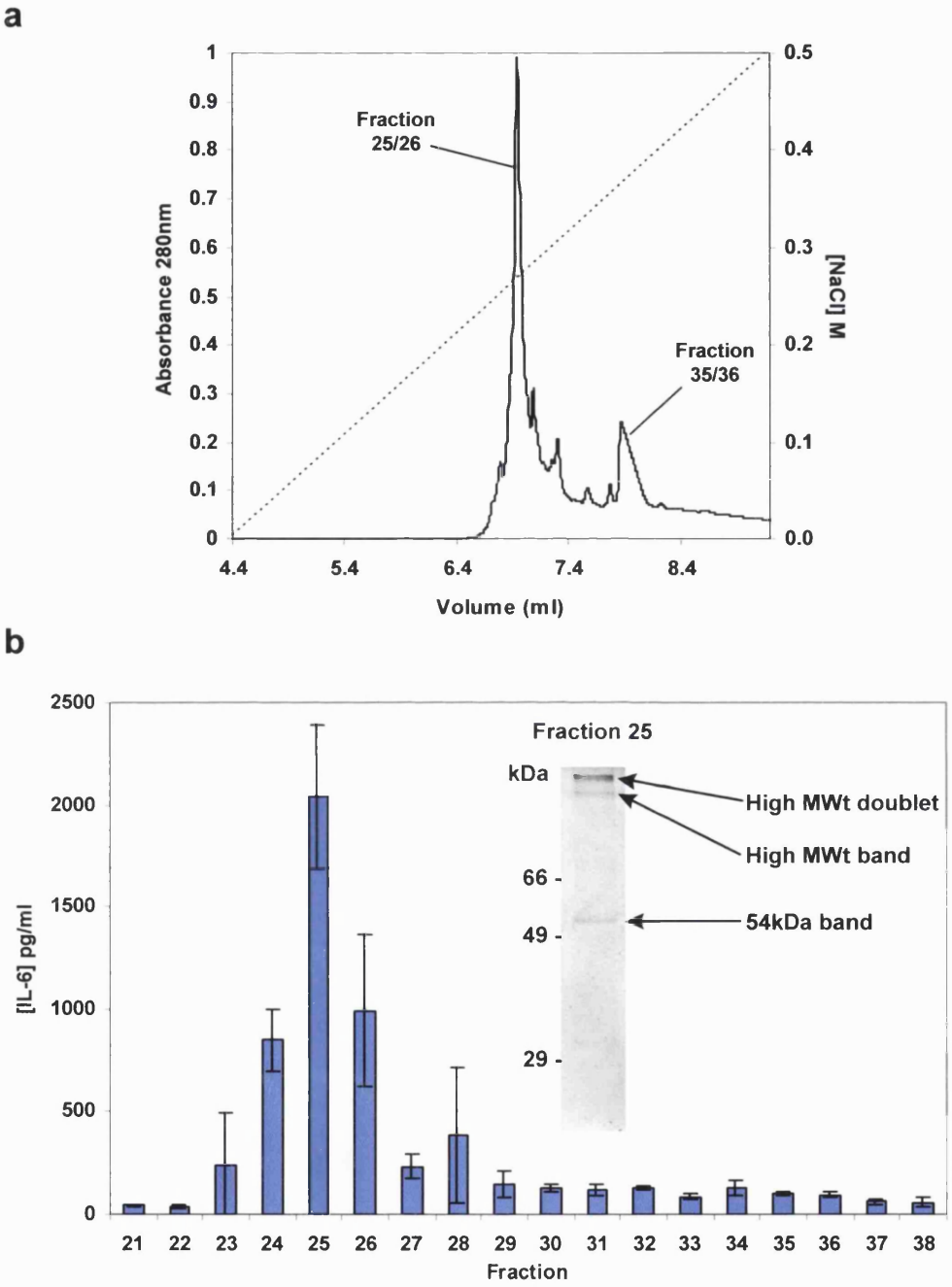

Figure 5.9 Concentration and further separation of fraction 15 using a MiniQ anion exchange column. Panel (a) shows the protein elution profile as absorbance at 280nm (solid black line), and the elution salt gradient (0-0.5M NaCl between 4.4 and 9.2ml) is shown by the black dotted line. Panel (b) shows the IL-6-inducing activity of fractions 21 to 38. No other fractions tested induced IL-6 secretion in PBMC (data not shown). Data is expressed as the mean and SD of triplicate cultures. Fractions were also separated on 12% SDS polyacrylamide gels and silver-stained. Only fraction 25 had protein bands visible with silver stain and this is shown as an insert in panel (b) (other fractions had no visible bands and are therefore not shown).

5.3.5.1 Further separation of fraction 25 (from Active Peak 1), using a Superdex 75 gel filtration column

Several peaks of protein were eluted from the gel filtration column, but the only IL-6-inducing activity was detected in fraction 7 (Figure 5.10a). SDS-PAGE analysis revealed that fraction 7 contained only the high molecular mass doublet (Figure 5.10b). No other bands were visible with silver stain in this, or any other fraction. The void volume of the Superdex 75 column was determined to be 0.9ml, corresponding to fraction 7 (Figure 5.11).

5.3.5.2 SDS-PAGE analysis of fraction 7

Several attempts were made to run the active fraction on 6% polyacrylamide gels so that the molecular mass of the protein could be more accurately estimated. Unfortunately no protein was recovered from the sample until the tube was boiled with reducing sample buffer, and even then only broadly smeared material was seen on the gel with silver stain (not shown), suggesting that the protein had degraded.

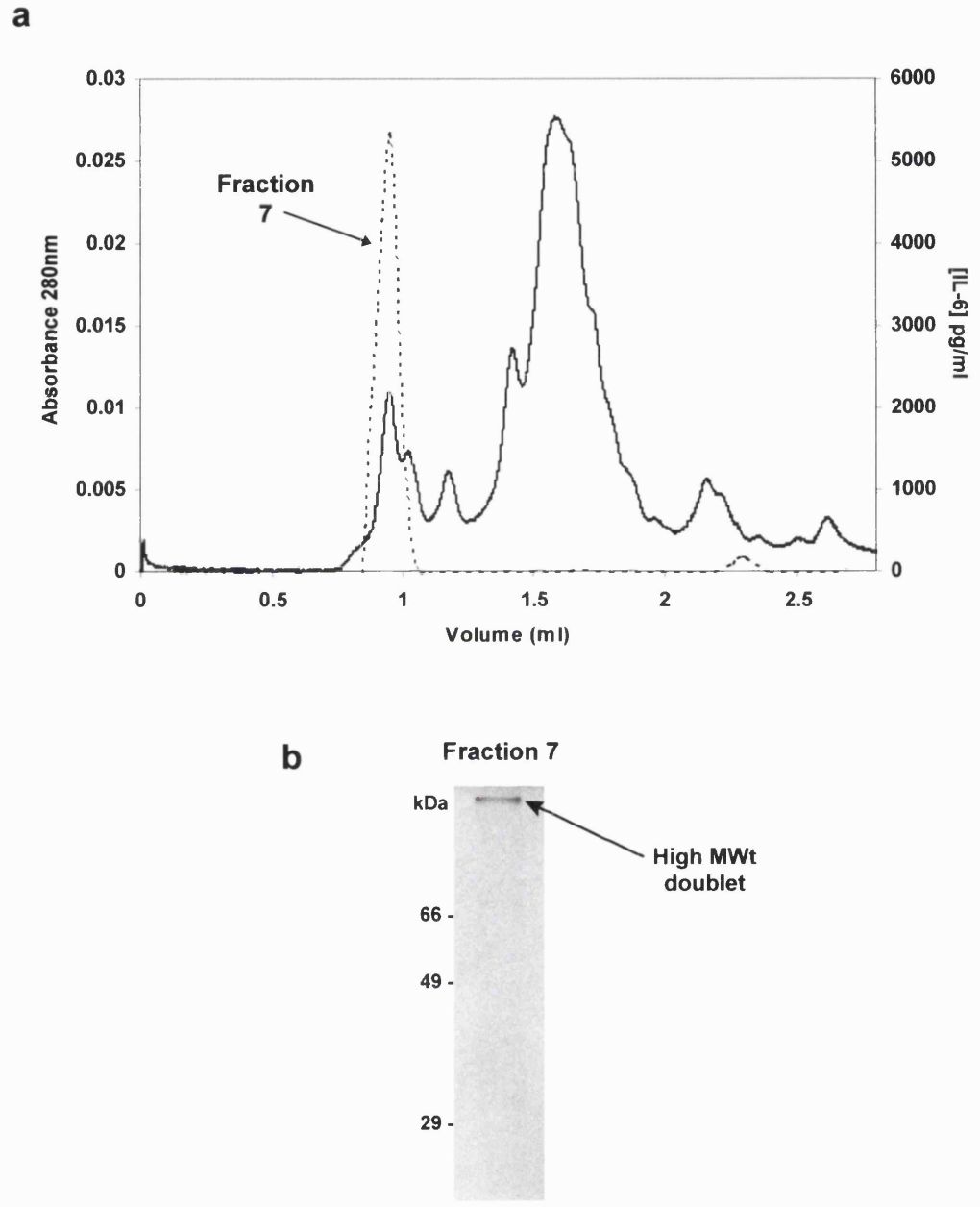

Figure 5.10 Further separation of fraction 25 using a Superdex 75 gel filtration column. Panel (a) shows the protein elution profile, measured as absorbance at 280nm (solid black line), and the IL-6-inducing activity of the fractions (dotted line). IL-6-inducing activity is expressed as the mean of triplicate cultures. Panel (b) shows the SDS-PAGE analysis of fraction 7. No other fractions revealed proteins visible by silver staining and are therefore not shown.

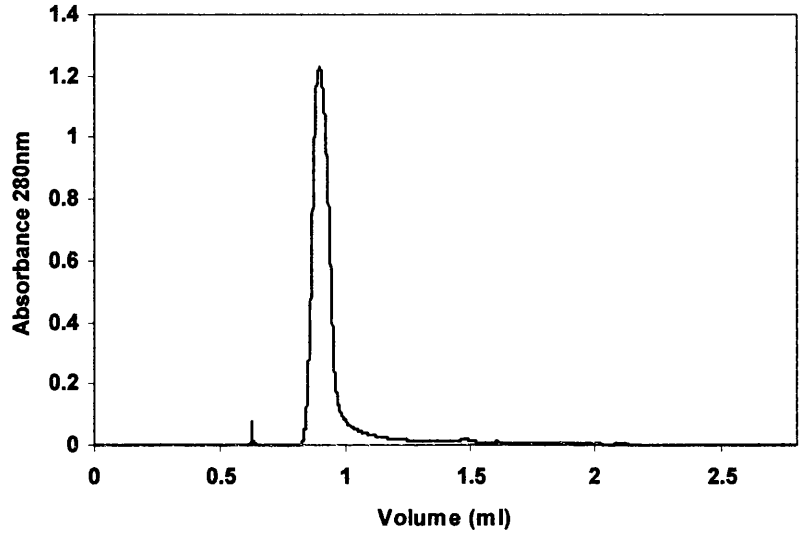

Figure 5.11 Void volume determination for the Sephadex 75 gel filtration column using Dextran Blue. The elution of this protein was monitored as absorbance at 280nm.

5.3.6 Repeat purification of the high molecular mass IL-6-inducing protein

S. sanguis EM was fractionated on a MA7Q column using a 0-1M NaCl gradient over 60ml, as had been done in the first attempt at purification. Although the elution profile (Figure 5.12a) was somewhat different from that obtained previously, SDS-PAGE analysis revealed that the high molecular mass protein was present in approximately the same fractions (Figure 5.12b), and hence had eluted at the same concentration of NaCl (between 0.08 and 0.2M). Fractions 10 to 17, all of which contained the high molecular mass protein, were concentrated and further separated by anion exchange chromatography using a MiniQ column. The chromatogram showed no distinct peaks (Figure 5.13) and SDS-PAGE revealed that the protein had eluted over 13 fractions despite the use of a shallower gradient (0-0.5M over 20 column volumes, as opposed to 12.5 column volumes) (gels not shown). Several of the fractions containing the protein of interest were separated on 6% SDS gels and electro-blotted (Figure 5.14). The molecular mass of the protein was calculated to be approximately 190kDa. Unfortunately no N-terminal sequence data were obtained for any of the fractions,

suggesting that either the protein was blocked at the N-terminus, or that there was insufficient protein to sequence.

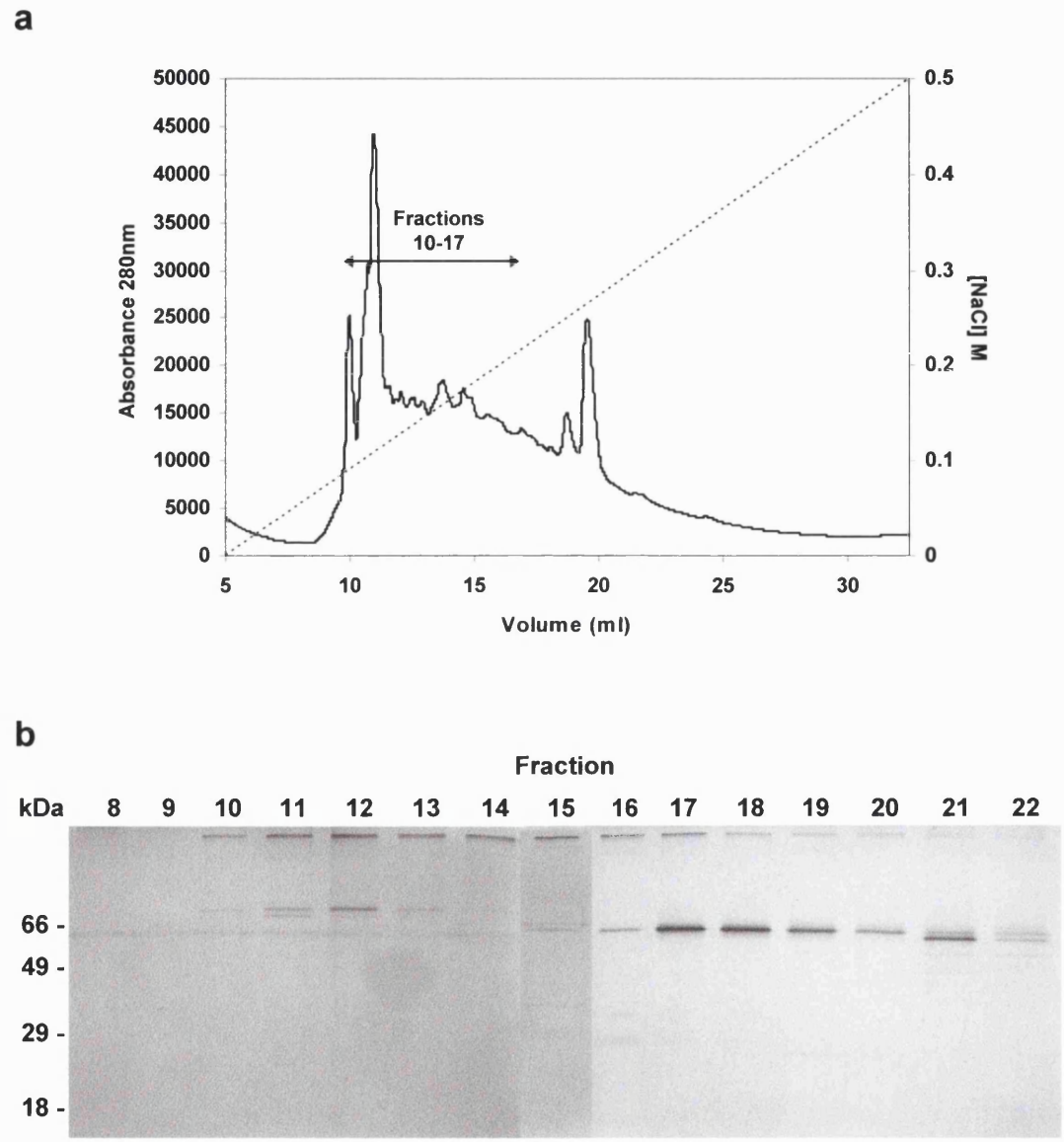

Figure 5.12 Fractionation of *S. sanguis* EM using a MA7Q anion exchange chromatography column. Proteins were eluted with a 0-1M NaCl gradient over 60ml. Panel (a) shows the elution profile, measured as absorbance at 280nm (solid line), and the gradient of NaCl (dotted line). Panel (b) shows the SDS-PAGE analysis of fractions 8 to 22 (only fractions containing the high molecular mass protein are shown here).

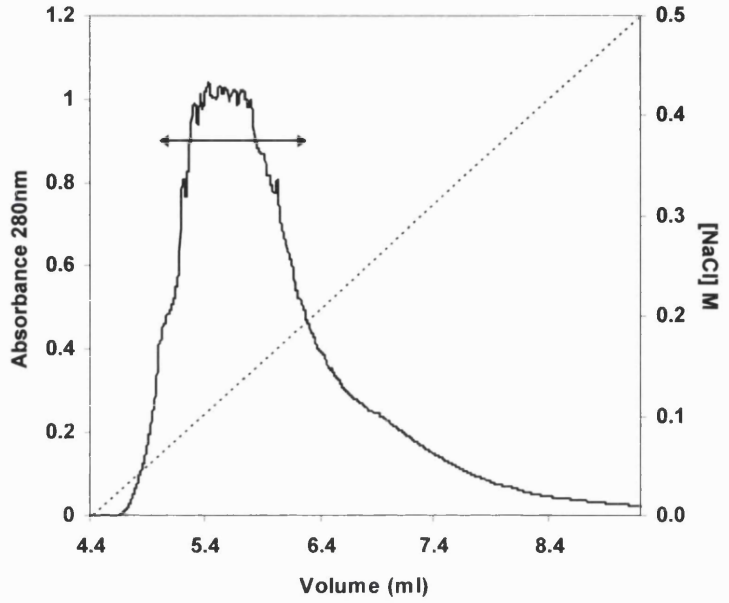

Figure 5.13 Concentration of the high molecular mass protein by anion exchange chromatography using a MiniQ column. The protein elution profile is shown as absorbance at 280nm (solid line) and the salt gradient (0-0.5M over 4.8ml) is shown as the dotted line. The double-headed arrow indicates the fractions containing the high molecular mass protein.

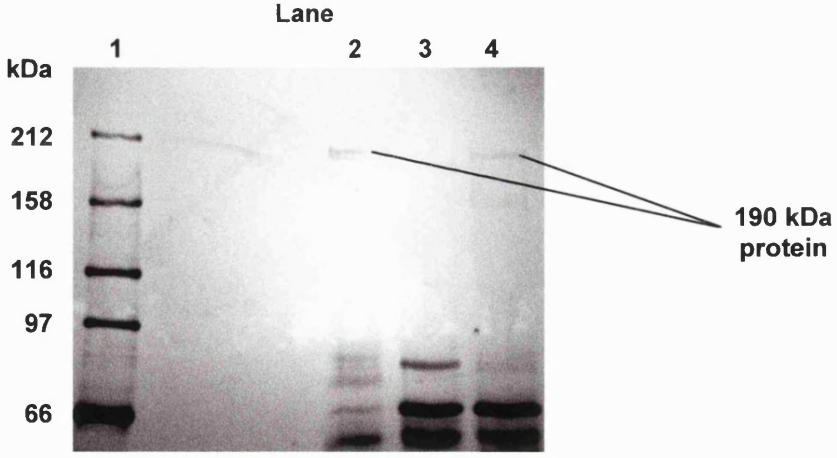

Figure 5.14 Electro-blotting of fractions containing the high molecular mass protein. Fractions were separated on 6% SDS gels and blotted onto PVDF membrane. The membrane was stained with 0.1% Coomassie Blue. Lane 1 shows the broad range molecular mass markers; Lanes 2-4 show three of the fractions containing the high molecular mass protein (fractions 9, 10 & 11).

5.3.7 Western blot analysis of the 190kDa protein using P1 and CshA antisera

SDS-PAGE analysis of a newly prepared batch of *S. sanguis* EM revealed the presence of high molecular mass proteins of approximately the same molecular mass as the 190kDa protein (Figure 5.15). The mutanolysin digests of *S. sanguis* and *S. gordonii* also contained several high molecular mass proteins; these were roughly the same mass as CshA or P1-like proteins. P1 antiserum detected a protein of 178 kDa in the *S. sanguis* mutanolysin extract, and a protein of 157 kDa in the *S. gordonii* extract (corresponding to the P1-like SspB protein which is 160kDa) (Figure 5.16a). No proteins were detected in the *S. sanguis* EM by the P1 antiserum. The CshA antiserum detected a pair of bands in both the *S. sanguis* and the *S. gordonii* mutanolysin extracts (Figure 5.16b). In both extracts the detected proteins were greater than 212 kDa and so their molecular mass could not be calculated from the molecular mass standards. As was the case with the P1 antiserum, no proteins were recognised in the *S. sanguis* EM with the CshA antiserum.

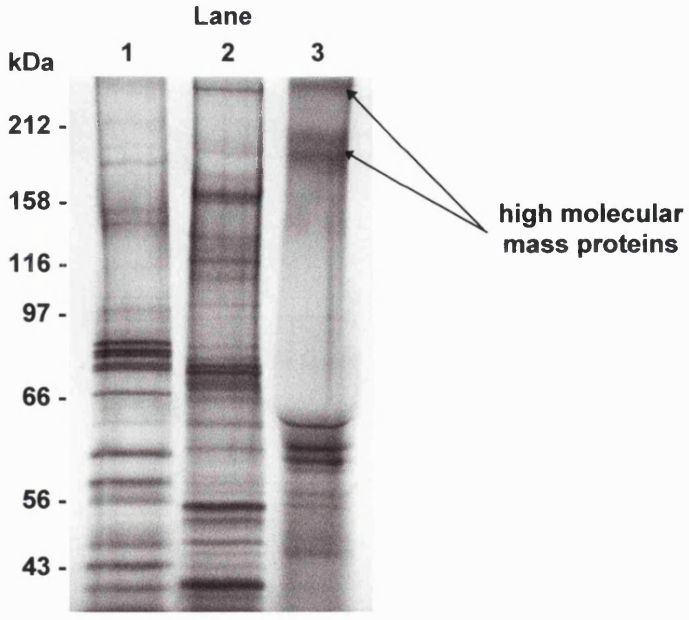

Figure 5.15 SDS-PAGE analysis of *S. sanguis* and *S. gordonii* mutanolysin digests and *S. sanguis* EM. 20μl of each mutanolysin digest and 30μg of *S. sanguis* EM were separated on 8% SDS polyacrylamide gels and silver-stained. Lane 1: *S. sanguis* mutanolysin digest; Lane 2: *S. gordonii* mutanolysin digest; Lane 3: *S. sanguis* EM.

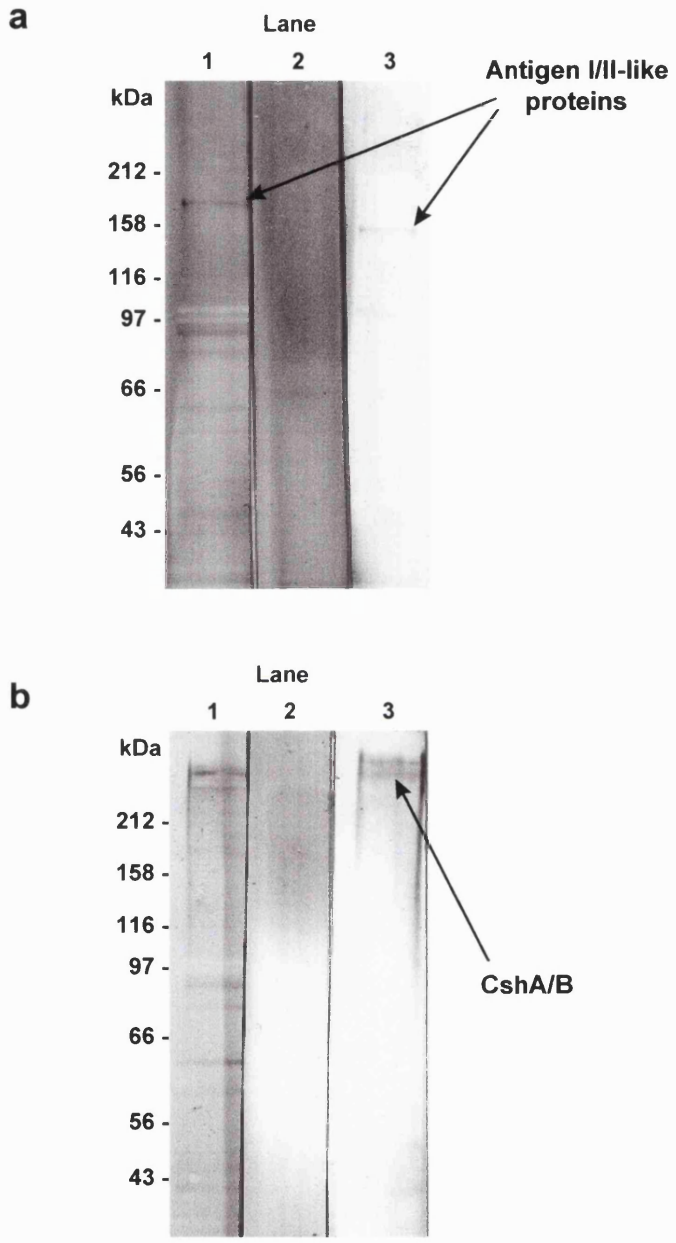

Figure 5.16 Western blot analysis of *S. sanguis* and *S. gordonii* mutanolysin digests and *S. sanguis* EM. 20μl of each mutanolysin digest and 30μg of *S. sanguis* EM were separated on 8% gels, electro-blotted onto PVDF membrane and probed with either P1 antiserum diluted 1 in 250 (panel (a)) or CshA antiserum diluted 1 in 500 (panel (b)). For both panels the lanes are as follows: Lane 1: *S. sanguis* mutanolysin digest; Lane 2: *S. sanguis* EM; Lane 3: *S. gordonii* mutanolysin digest.

5.4 Discussion

The previous two chapters described an extract of secreted material from *S. sanguis* (termed "exported material", or "EM") which was shown to possess potent cytokine-inducing activity. This activity was determined to be due to proteinaceous components, and this chapter documents attempts made to purify the active proteins.

The binding affinity of triazine dyes for proteins was discovered by accident in the 1960s during a gel filtration purification where Cibacron Blue had been attached to a high molecular mass dextran for use as a visible void-volume marker. In this case the dye bound yeast pyruvate kinase, but was later discovered to bind other enzymes, particularly dehydrogenases. Other triazine dyes were investigated and found to exhibit similar affinities, some being strongly selective for particular proteins. For example, reactive red 120 selectively binds NADP-binding proteins (Dean and Watson, 1979). The inclusion of dye-affinity chromatography in protein purification procedures has been increasing ever since, probably due to the cheapness and stability of the dyes (Lowe and Pearson, 1984). The development of computer-designed synthetic mimetic ligands has eliminated dye-leakage, and increased the specificity of interactions, making the purification of bioactive molecules a more realistic prospect (Lowe *et al.* 1992). For example, a recent report documents the use of a mimetic dye-ligand matrix (Mimetic Green 1 A6XL) to purify biologically active recombinant human interferon alpha to near homogeneity in a single step (Swaminathan and Khanna, 1999).

As a first step in purifying cytokine-inducing proteins from *S. sanguis* EM, use was made of a commercially available set of dye columns (Sigma). Of the dye columns screened, only column C-1535 (Cibacron blue) bound any protein from the EM. The protein, of approximately 56kDa, failed to be eluted from the column with 2.5M NaCl. This may reflect a greater importance of hydrophobic interactions than ionic interactions in the protein-dye binding, (although if actual affinity interactions were involved, the binding may have been strong enough to resist elution by NaCl). Thus elution might have been helped by the addition of solvents such as ethanol or ethylene glycol, or changes in temperature. Unfortunately, by the time it was discovered that the

protein had not been eluted (the eluate had to be concentrated first before running on a gel), the dye column had been regenerated and the protein lost. To establish whether the 56kDa protein was solely responsible for the cytokine-inducing activity of the EM, samples of the unbound protein from the C-1535 column (i.e. samples from which the 56kDa protein had been removed) were tested in routine PBMC assays. No IL-6-inducing activity was recovered, but the control sample (unbound protein from a different dye column that had not retained the 56kDa or any other protein) was also inactive, so no conclusion could be drawn about the role of the 56kDa protein. The dye-affinity chromatography resulted in considerable dilution of the samples, meaning that they could not be directly tested for activity without having been concentrated first, and it is possible that the activity was lost at this stage. Irrespective of whether the loss of activity was due to the purification procedure itself or the subsequent concentration, dye-affinity chromatography seemed to have limited potential in the purification of EM proteins, and so it was decided to investigate other techniques of purification.

Ion exchange chromatography proved to be a much more fruitful approach. To aid understanding, the reader should refer to the flow diagram (Figure 5.2) which has been included so that the discussion of the purification schemes can be followed more readily. Initially, 20mg of *S. sanguis* EM was fractionated by anion exchange chromatography using a MA7Q column with a 0-1.0M salt gradient. The proteins separated into several well-defined peaks with most material being eluted at <0.25M NaCl. Two main peaks of IL-6-inducing activity were recovered: fractions 15 & 16 (Active Peak 1), and fractions 19 and 20 (Active Peak 2), both of which eluted at less than 0.25M NaCl. The most active fractions (19 & 20) contained several proteins which could have been responsible for the IL-6-inducing activity. Those proteins that were unique to fractions 19 and 20, and therefore the most likely candidates for activity, were present only in small amounts. The predominant protein in these fractions (a 60kDa protein) was thought to be a less likely candidate for the activity since it was present in larger amounts in less-active fractions. However, the presence of other unidentified proteins (which may possibly have been inhibitory to the 60kDa protein) in these less-active fractions threw doubt on the assumption that the 60kDa protein was

not the active component. In light of this uncertainty, fractions 17 and 18, containing large amounts of the 60kDa protein, were pooled with fractions 19 and 20 for further purification.

The next step in the purification procedure was aimed at both concentrating and further separating the proteins in fractions 17 to 20. To achieve this, the entire sample was injected onto a MiniQ anion exchange chromatography column, and the proteins eluted using a shallower salt gradient. The elution profile, measured as absorbance at 280nm, revealed that the proteins had been successfully separated into several distinct peaks. However, one of the peaks absorbing strongly at 280nm did not appear to contain any proteins on examination by SDS-PAGE. Conversely, the fractions containing the most abundant protein (the 60kDa protein, visible in fractions 22-24) showed low absorbance at 280nm. The latter may be a result of the protein(s) containing low levels of tyrosine and tryptophan residues, and in fact measurement of absorbance at 214nm, which is a measure of peptide bonds and is less dependent on amino acid composition, suggested that fractions 22-24 did contain substantial amounts of polypeptides. The peak with a strong absorbance at 280nm but with an apparent absence of protein, may have contained very small amounts of a protein that was a very strong absorber at 280nm, for example a tyrosine or tryptophan rich protein. Such protein might be visible with silver stain if larger amounts were separated on SDS polyacrylamide gels, but as samples were precious this was not attempted.

The IL-6-inducing activity was almost exclusively present in fractions 22 to 24, which contained a number of possible candidates for this activity. Again the question of whether the 60kDa protein was the active component arose. It was present in the largest amount in fractions 22 and gradually decreased through fractions 23 to 25, but the activity of the fractions (0.2µl of each fraction tested) did not seem to follow the same pattern: the activity of fraction 22 was no higher than that of fraction 23. To confirm that this was not simply due to the PBMC reaching their maximum response capacity for this protein, a ten-fold dilution of each fraction was re-tested for activity.

The activity was thus pin-pointed to fraction 23, and the 60kDa protein rejected as the IL-6-inducing component, as it's presence did not appear to correlate with activity.

The only protein unique to the active fraction was a 23kDa protein, so this was subjected to N-terminal sequence analysis. Analysis gave a sequence of 18 amino acids which were used to search GenBank using the NCBI BLAST network. This protein was determined to be 88% homologous (in terms of amino acid identities) to the *S. mutans* and *S. pneumoniae* manganese-dependent superoxide dismutases (SOD). The database searches did not detect homology with the *S. sanguis* SOD protein, although further investigation revealed that the sequence on the database for this protein was deduced from the nucleotide sequence of an internal fragment representing only 85% of the gene (Poyart *et al.* 1998). The database sequence started from the equivalent of residue 18 of the *S. mutans* SOD protein, and so a match for our protein and *S. sanguis* SOD would not be expected to be found. Another search confirmed that *S. sanguis* SOD was 80% homologous to that of *S. mutans*.

To determine whether SOD could have been responsible for the IL-6-inducing activity, a commercial preparation was assessed for the ability to induce IL-6 secretion by PBMC. SOD from *S. sanguis* was unavailable, so a manganese-dependent SOD from *B. stearothermophilus* was used instead (the amino acid sequences of the SOD proteins from these two organisms were determined, by a GenBank search using the NCBI BLAST network, to be 68% homologous in terms of amino acid similarities and an identity of 57%). Although the protein was able to induce IL-6 secretion, the addition of polymyxin B completely blocked this activity, suggesting that the preparation may have been contaminated with LPS, and so this work was not taken further. It is possible that the commercial SOD did have the capacity to induce cytokine synthesis , but had become inactive as a result of storage conditions (freeze-thawing), leaving activity to contaminating LPS or peptidoglycan.

Superoxide dismutase is present in the cytoplasm of most bacteria, its function being to destroy endogenous oxygen radicals that are toxic to biological systems. In addition,

the number of bacterial species reported to secrete SOD is growing; in particular there have been several reports of Gram-negative organisms expressing copper-zinc-dependent SODs with leader peptides directing secretion directly into the periplasm (Kroll *et al.* 1995; Langford *et al.* 1996; Imlay and Imlay, 1996), and in some bacteria this has been linked to pathogenicity (Farrant *et al.* 1997). Secretion of SOD into culture supernatant has been demonstrated in *Mycobacterium tuberculosis*, *M. bovis* BCG as well as the opportunistic pathogens *M. kansasii* and *M. fortuitum* (Raynaud *et al.* 1998). In this latter report the secretion of SOD was suggested to be linked to virulence as the non-pathogenic species, *M. phlei* and *M. smegmatis*, did not secrete this protein. The only streptococcal species reported to secrete SOD is *S. pyogenes*, but with no evidence of a leader sequence, it is either secreted by a non-conventional mechanism or was released into the culture supernatant by lysed bacteria (something that was not clarified in this report) (Gerlach *et al.* 1998). Since there is no evidence of extracellular SOD expression in any other streptococcal species, it is unlikely (although not impossible) that *S. sanguis* secretes this enzyme. Even if *S. sanguis* does not actually secrete SOD, cytoplasmic SOD may still be released *in vivo* due to bacterial lysis. It would be interesting to investigate whether expression of this enzyme is regulated by growth in different environments, perhaps reflecting a movement from the oral environment to that of the heart. Aerobic growth of *S. pneumoniae* has recently been reported to result in increased levels of cytosolic MnSOD compared to anaerobic growth (Yesilkaya *et al.* 2000).

The initial fractionation of EM using a MA7Q anion exchange chromatography column separated the proteins into several peaks of IL-6-inducing activity, so after the further purification of fractions 17, 18, 19 and 20 (Active Peak 2), the investigation turned to the next most active peak, Active Peak 1. Although the fractions constituting Active Peak 1 (15 and 16) contained several proteins, a protein of approximately 30kDa, which appeared to be unique to these fractions, was thought the most likely to be responsible for the IL-6-inducing activity. This protein band was extremely feint in fraction 16, so fraction 15 was chosen for further purification.

For the next step in the purification of fraction 15, a similar procedure was adopted to that used previously for Active Peak 2 (fractions 17 – 20), namely the use of a MiniQ column to concentrate and further separate the proteins. A shallower elution gradient was used than for the initial MA7Q anion exchange chromatography and this appeared to separate the proteins in fraction 15 into several peaks. The IL-6-inducing activity was found to correspond to the first major peak, fraction 25, eluting at approximately 0.26M NaCl. SDS-PAGE analysis of the active fraction revealed three possible candidates for this activity: a high molecular mass protein doublet, a single band also of high molecular mass, and a 54kDa protein. There was no evidence of the 30kDa protein in this, or any of the other fractions. It is possible that the 30kDa protein may not have been eluted and remained bound to the column. During the initial fractionation with the MA7Q column, there would have been a large amount of protein competing for binding to the charged groups on the column matrix. During the subsequent purification step with the MiniQ column much less protein was competing for these charged groups, and so previously weakly-binding proteins may have been able to bind more strongly, requiring a higher concentration of salt to effect elution. If this was the case, the maximum concentration used in the salt gradient (0.5M) may no longer have been sufficient to disrupt binding. The observation that the proteins in fraction 15 had been eluted from the MA7Q column at 0.16-0.18M NaCl, but that elution of these same proteins from the MiniQ column required upwards of 0.26M NaCl, adds weight to this hypothesis. Alternatively, it is possible that the 30kDa protein was simply too dilute to be seen on these gels, in which case it may have been present in fraction 25, and may well have been responsible for the activity of this fraction.

Even if the 30kDa protein was present in fraction 25, it was shown to be unlikely to be the active component after further fractionation of the proteins in this fraction. Gel filtration chromatography, using a Superdex 75 column to separate the proteins on the basis of their size, produced several peaks of protein, but the IL-6-inducing activity was confined to fraction 7. The elution of this activity in the void volume was consistent with proteins which exceeded the 100kDa exclusion limit of the Superdex 75 column, and indeed the active fraction contained the high molecular mass protein doublet, as

determined by SDS PAGE. The other high molecular mass protein should also have eluted in the void volume but there was no evidence of this protein in any of the fractions when visualised on SDS polyacrylamide gels with silver stain. Neither was the 54kDa protein visible in any of the fractions. Since the gel filtration procedure resulted in dilution of the proteins, it is possible that both these proteins were too dilute to be seen. Alternatively they may have actually been adsorbed by the column matrix through ionic or hydrophobic interactions; this has been reported to occur with certain proteins under particular buffer conditions (Belew and Fohlman, 1978).

Attempts were made to obtain a more accurate estimation of the mass of the high molecular mass protein doublet by running fraction 7 on low percentage SDS polyacrylamide gels. Unfortunately significant degradation had occurred and the protein was lost.

A second attempt was made to purify the high molecular mass protein using the same purification procedure: fractionation using a MA7Q column was followed by concentration and further separation using a MiniQ column. The high molecular mass protein was electro-blotted for N-terminal sequencing, but no sequence was obtained either due to the N-terminus being blocked, or an insufficiency of protein. However, the molecular mass of the protein was calculated from the protein standards and determined to be 190kDa.

Due to the large size of the protein, it was possible that it may have been related to either the streptococcal antigen I/II family of adhesins, or to the fibronectin-binding proteins of *S. gordonii*, CshA/CshB. CshA is a 254kDa adhesin which is localised in the surface fibrils of *S. gordonii* (McNab *et al.* 1999), and antigenically-related polypeptides are expressed by *S. sanguis* (McNab *et al.* 1995). The antigen I/II proteins are probably the best characterised adhesins of oral streptococci and were first identified in *S. mutans* (Russell and Lehner, 1978). It is now accepted that antigen I/II-like polypeptides are expressed by almost all species of oral commensal streptococci, examples of which include *S. gordonii* SspA/SspB, *S. sobrinus* SpaA, and *S. mutans*

SpaP, P1 and PAc (Jenkinson and Demuth, 1997a). They have multiple ligand-binding sites which facilitate binding to human salivary glycoproteins, other microbial cells, and calcium. Antibodies against PAc have been identified in the sera of patients with *S. mutans* endocarditis (Russell *et al.* 1992), and a 180kDa antigen from *S. oralis* detected by immunoblotting with sera from endocarditis cases was found to be highly homologous to PAc and other antigen I/II proteins (Burnie *et al.* 1996).

Both P1 (antigen I/II) antiserum and CshA antiserum were available, and so it was decided to perform Western blotting analysis to determine whether the 190kDa protein could be detected by these antibodies. All the fractions containing large amounts of the 190kDa protein had been used in the attempt to obtain N-terminal sequence data. Other fractions that had been shown previously to contain smaller amounts of this protein were examined by SDS-PAGE, but no 190kDa protein was visible, suggesting that degradation had occurred. As an alternative, whole, unpurified *S. sanguis* EM was probed with the antisera, after first establishing the presence of undegraded high molecular mass proteins. Digestion of whole *S. gordonii* with mutanolysin has been used to release both intact SspA/SspB and CshA/CshB (McNab and Jenkinson, 1998), and so mutanolysin extracts from *S. sanguis* and *S. gordonii* were prepared for use as controls in the Western blot analysis. Although the P1 antibodies detected antigen I/II-like proteins in both the control mutanolysin extracts, no proteins were detected in the *S. sanguis* EM. Neither were any proteins detected in the EM by the CshA antiserum, although protein doublets were detected in both the control extracts. *S. sanguis* EM is therefore unlikely to contain adhesins related to either CshA or the antigen I/II proteins, and consequently it is unlikely that the purified 190kDa is related to these oral streptococcal adhesins.

The main problem encountered in the purification of cytokine-inducing proteins from EM was that these proteins were present in very small amounts, and subsequently the purification of enough material to obtain N-terminal sequence data proved difficult (with the exception of the SOD protein). With more time, the purification could be repeated on a larger scale, but it was decided instead to turn to proteins secreted from

planktonically-grown bacteria, as scaling up would be considerably easier. The next chapter is devoted to this alternative approach.

CHAPTER 6

ISOLATION OF CYTOKINE-MODULATING PROTEINS FROM *S. sanguis* CULTURE SUPERNATANT

6.1 Introduction

Chapter 4 described the biological activity of exported material extracted from *S. sanguis* grown as a biofilm in the presence of blood. This material contained a protein or proteins capable of inducing IL-6 secretion by PMBC, and the attempts made to purify the active components were described in Chapter 5. However, the cytokine-inducing proteins were present in very small amounts, insufficient for N-terminal sequencing. Growth and harvesting of bacteria on agar plates is extremely labour-intensive and the number of plates that would be needed to produce sufficient protein for N-terminal sequencing precluded this method of production. Much larger amounts of bacteria can be produced relatively quickly in liquid culture, but since the growth state of bacteria is known to affect protein expression (Costerton *et al.* 1995), a preliminary study was carried out to look at the culture supernatants of planktonically-grown *S. sanguis* for the ability to induce IL-6 secretion by PBMC.

6.2 Materials and methods

6.2.1 Pilot study

6.2.1.1 Bacterial culture

S. sanguis NCTC 10904 was cultured from glycerol stocks onto Wilkins Chalgren agar plates and incubated overnight at 37°C in an anaerobic chamber. Growth from these plates was used to inoculate 10ml of Wilkins Chalgren broth (Oxoid, Basingstoke). Broth cultures were incubated without agitation at 37°C overnight in an anaerobic chamber. The following types of liquid media were prepared:-

1. Wilkins Chalgren broth (WCB)

2. Ultrafiltrated Wilkins Chalgren broth. Wilkins Chalgren broth was pre-filtered using an Amicon ultrafiltration spin-cell with a 10 kDa molecular mass cut-off membrane as described in Chapter 2, section 2.8.2 to remove large proteins before autoclaving (referred to from this point on as WCB*). This would make it easier to distinguish bacterial proteins from proteins originating from the culture medium.

3. Tryptone Soya Broth supplemented with 5g/L yeast extract (TSBY).

600ml of each type of medium was inoculated with a 10ml overnight culture of *S. sanguis* and incubated at 37°C without agitation for 16 hours. Controls of medium with no bacteria were prepared in parallel. Bacteria or control cultures were centrifuged at 10,000 x g in a Sorvall refrigerated centrifuge with a GSA rotor for 30 minutes at 4°C in sterile 250ml Nalgene centrifuge pots. The resulting supernatants were re-centrifuged as described above and then filtered through 0.45μm PVDF filters. Bacterial pellets were freeze-dried and the yield of dry weight/ml bacterial culture calculated for each type of medium.

6.2.1.2 Ammonium sulphate precipitation

Protein from the supernatants was precipitated using solid ammonium sulphate as described in Chapter 2, section 2.8.1. Precipitates were harvested by centrifugation for 30 minutes at 10,000 x g (Sorvall centrifuge with GSA rotor) at 4°C and solubilised in approximately 5ml pyrogen-free water (Associates of Cape Cod) before dialysing against 20 x 5 litre changes of distilled water at 4°C using dialysis membrane with a 3.5 kDa molecular weight cut-off (Spectrapor).

6.2.1.3 SDS-PAGE

Samples of each protein preparation were separated by SDS PAGE on 12% gels as described in Chapter 2, section 2.3 and the proteins visualised by silver staining as described in section 2.3.3. Samples from bacterial supernatants were also visualised with colloidal blue stain (Sigma) as described in section 2.3.4.

6.2.1.4 Assessment of the cytokine-inducing activity of the *S. sanguis* culture supernatant protein preparations

A 10 x PBS stock was added to the protein to bring the final concentration to 1 x PBS. For each protein preparation a series of ten-fold dilutions was made in PBS. PBMC were prepared and plated out as described in Chapter 2, section 2.5. Various concentrations of the proteins were added to each well in triplicate. Equal volumes of control protein preparations (from cultures without bacteria) were tested alongside those derived from bacterial supernatants to give some idea of how much of the IL-6-inducing activity was due to proteins in the medium rather than bacterial protein. In all experiments the level of IL-6 secreted into the medium was measured by a two-site ELISA as described previously (Chapter 2, section 2.6).

6.2.2 Large-scale preparation of bacterial culture supernatant protein

Two x 1.2 litres of TSBY were prepared as described previously. Each was inoculated with 24ml of an overnight culture of *S. sanguis* (to give a 1 in 50 dilution of bacteria), and incubated at 37°C without shaking for 16 hours. The culture supernatant was collected and the proteins precipited using ammonium sulphate, as described for the pilot study (sections 6.2.1.1 and 6.2.1.2). Protein from the culture supernatant was visualised on 12% gels as described in section 6.2.1.3, and this was used to assess the reproducibility of different batches.

6.2.2.1 Assessment of the cytokine-inducing activity of large scale *S. sanguis* culture supernatant protein

The cytokine-inducing activity of the protein preparations was tested in a routine PBMC assay as described in section 6.2.1.4. To determine whether the cytokine-inducing components were proteinaceous, samples of the protein were heated for 30 minutes in a boiling water-bath before testing in the routine PBMC assay. The activity of heat-treated protein was compared to a control sample of untreated protein.

6.2.3 Fractionation of protein preparations from planktonically-grown *S. sanguis* using anion and cation exchange chromatography cartridges

To determine whether anion or cation exchange chromatography would provide a useful method for purification of individual proteins from the supernatant obtained from planktonically grown *S. sanguis*, rapid separation of a small amount of the protein preparation was carried out using Bio-Rad ion exchange chromatography cartridges with 5ml bed volumes. Equilibration buffers (referred to as buffer A) of 20mM Tris pH 8.5 and 20mM MOPS pH 6.0 were prepared for the Econopac Q anion exchange chromatography cartridge and the Econopac S cation exchange chromatography cartridge respectively. For each cartridge an elution buffer consisting of 1M NaCl in the appropriate equilibration buffer was prepared (referred to as buffer B). Samples of the proteins obtained by ammonium sulphate precipitation were prepared by dilution with 10 x solutions of the appropriate equilibration buffer to a final concentration of 1 x equilibration buffer. All buffers and protein solutions were applied to the cartridges at a flow rate of 1-3ml/minute using a 10ml syringe. Cartridges were first cleaned by the application of 10ml buffer B (1M NaCl), and then equilibrated with 10 column volumes of buffer A. After equilibration, 10ml of appropriately buffered protein preparation (containing 40mg protein) was loaded onto each cartridge and the unbound material collected (labelled "run-off I"). 5 column volumes of buffer A were run through to wash off any remaining unbound protein and this was collected (labelled "run-off II"). 10ml of buffer B was loaded onto the column and 2ml fractions collected. This was followed by 20ml buffer A during which 2ml fractions were collected, and then a further 30ml buffer A which was not collected.

6.2.3.1 SDS-PAGE analysis

Samples of whole supernatant, run-offs and fractions were separated by SDS PAGE on 12% gels and the proteins visualised by silver staining as described in Chapter 2, section 2.3.

6.2.3.2 Assessment of IL-6-inducing activity

50µl of the unfractionated protein preparation, the run-offs and fractions were tested in triplicate for their ability to induce PBMC to secrete IL-6 in the routine 1ml PBMC bioassay. The resulting levels of IL-6 in assay supernatants were measured by two-site ELISA (described in Chapter 2, section 2.6).

6.2.4 Large-scale fractionation of precipitated culture supernatant proteins from *S. sanguis* by cation exchange chromatography.

A Bio-Rad S20 cation exchange HPLC column was used on a Bio-Rad HRLC system. The column was prepared as described in Chapter 2, section 2.8.3.1. The culture supernatant protein was diluted in 10 x buffer A (0.2M MOPS pH 6.0) to a final concentration of 1 x buffer A, dispensed into Eppendorfs and centrifuged at 14,000 x g for 10 minutes at 4°C to remove particulates. Initially a 50ml Superloop was used to load just 40ml of protein solution (equivalent to a total of 160mg protein) onto the S20 column. The run-off containing unbound protein was collected (approximately 40ml). The column was then washed with 10 column volumes of buffer A, the first 100ml of which was collected. Proteins were eluted with 10 column volumes of buffer B (buffer A plus 1M NaCl) during which 4ml fractions were collected. Finally, the column was washed with 10 column volumes of buffer A and 4ml fractions were again collected. Due to the large number of fractions collected, samples were pooled in threes to give 12ml fractions. However, the first six fractions collected immediately after the column was changed from buffer B to buffer A were kept without pooling.

The remaining 120ml of the protein preparation (equivalent to 480mg protein) was loaded onto the S20 column in three portions using the Superloop, and eluted using the same method as for the first 40ml. To determine if the column had been overloaded, 40ml of run-off containing unbound protein was re-loaded onto the re-equilibrated column, and the whole elution procedure run through again.

6.2.4.1 SDS-PAGE analysis

36µl of each fraction was separated by SDS PAGE using 12% resolving gels and visualised by silver staining as described in Chapter 2, section 2.3.

6.2.4.2 Assessment of IL-6-inducing activity

To test the fractions for IL-6-inducing activity, PBMC were isolated from pooled donor "buffy coat residue blood" rather than single donor "buffy coat blood". The reason for this was because single donor blood proved difficult to obtain at short notice, and in purifications such as these, the speed of progress is important if one is not to suffer losses in activity while fractions wait to be assayed. In addition, several results had suggested that donor variation could be a problem in some experiments. It was found that blood from two different donors gave completely different results. The use of pooled donor "buffy coat residue blood" would minimise the possibility of donor variation affecting the reproducibility of results. 50µl of each fraction was tested in triplicate for IL-6-inducing activity. The level of IL-6 secreted into the supernatant was measured by two-site ELISA as described previously (Chapter 2, section 2.6).

6.2.5 Concentration of IL-6-inducing fractions

Fractions 25, 26 and 27 were pooled and concentrated six-fold by ultrafiltration using an Amicon spin-cell with a 10kDa cut-off filter membrane (YM10) as described in Chapter 2, section 2.8.2. The pooled fractions were then further concentrated using an Amicon Microcon 10 (10kDa cut-off membrane) to a final volume of just under 100µl, giving a total concentration factor of 120.

6.2.6 Gel filtration chromatography of the IL-6 inducing fractions

A Superdex 75 gel filtration column (details of which are given in Chapter 2, section 2.3.3.2) was used on a SMART system (Pharmacia), with PBS as the buffer. During the runs the absorbance at 280nm was monitored, and 50µl fractions were collected. The sample was fractionated in two runs and duplicate fractions pooled. 2µl of each fraction was run on a 12% SDS-PAGE gel and visualised with silver stain. IL-6-

inducing activity was assessed using the routine PBMC assay with 0.5µl of each fraction tested in triplicate.

6.2.6.1 SDS-PAGE and electro-blotting of the active fraction for N-terminal sequencing

To minimise the loss of protein, concentration of the active fraction was not carried out. Instead the whole fraction was run in two lanes of a 1.5mm thick SDS-PAGE gel consisting of 8% acrylamide, as described in Chapter 2, section 2.3. The proteins were blotted onto PVDF membrane using the CAPS buffer system (section 2.4.2), and stained for 5 minutes with 0.1% coomassie blue as described (section 2.4.2.1).

6.2.7 Fractionation of proteins which failed to bind to the S20 cation exchange HPLC column

Since a great many proteins from the culture supernatant preparation did not bind to the S20 cation exchange HPLC column, and retained substantial IL-6-inducing activity, it was decided to attempt to fractionate these proteins and isolate the active component(s). These proteins have been defined as the S20 column run-off proteins in the following sections.

6.2.7.1 SDS-PAGE analysis of whole *S. sanguis* culture supernatant protein, and the S20 column run-off protein

20µl of both whole *S. sanguis* culture supernatant protein and the S20 column run-off protein was run on a 12% SDS-PAGE gel and silver stained.

6.2.7.2 Preparation of S20 column run-off proteins for anion exchange HPLC

A total of 160ml protein that didn't bind to the S20 HPLC column had been collected. This was concentrated to 40ml in an Amicon ultrafiltration spin cell with a 10kDa cut-off membrane, as described in Chapter 2, section 2.8.2. The sample was then flushed through the same membrane several times with distilled water to bring the

concentration of buffer A (20mM MOPS pH 6.0) down to less than 50μM, and the sample to a final volume of 80ml. A stock solution of 0.2M Tris pH 8.5 (10 x buffer A) was added to bring the protein solution to a final buffer concentration of 20mM Tris. The protein was then dispensed into Eppendorfs and centrifuged at 14,000 x g for 10 minutes at 4°C to remove particulates.

6.2.7.3 Fractionation of S20 column run-off proteins by anion exchange HPLC

A Bio-Rad Q20 anion exchange HPLC column was used on a Bio-Rad HRLC system. The column was prepared as described in Chapter 2, section 2.8.3.1. A 50ml Superloop was used to load 45ml of protein solution onto the Q20 column. The run-off containing unbound protein was collected (approximately 40ml). The column was then washed with 100ml buffer A. Proteins were eluted with a 0-1M NaCl gradient (buffer B) over 200ml (10 column volumes) during which 4ml fractions were collected. Finally, the column was washed with 10 column volumes of buffer A. The whole procedure was repeated for the remaining 45ml protein. Because of the large number of fractions collected (in excess of 50), fractions were pooled in pairs for the purposes of SDS-PAGE analysis and for the assessment of activity.

6.2.7.3.1 SDS-PAGE analysis

36μl from each of the pooled fractions was separated on 12% SDS-PAGE gels and silver stained. Samples of the unfractionated S20 column run-off protein and protein that failed to bind to the Q20 HPLC column were also run.

6.2.7.3.2 Assessment of IL-6-inducing activity

PBMC were isolated from mixed-donor buffy coat blood and a routine PBMC assay set up, as described in Chapter 2, section 2.5. A small amount of each pooled fraction was diluted in sterile PBS so that the equivalent of 0.5μl could be added to the assay wells in 20μl aliquots for increased pipetting accuracy. All fractions were tested in triplicate.

The level of IL-6 secreted by the PBMC was measured by two-site ELISA, as described in Chapter 2, section 2.6.

6.3 Results

6.3.1 Pilot study

6.3.1.1 Effect of different culture media on bacterial growth yields

Table 6.3.1 shows the yields of bacteria obtained from each of the three types of liquid medium. Growth in TSBY gave the highest yield.

Culture medium	Total dry weight of bacteria (mg)	Yield (dry weight of bacteria/volume of culture) (g/L)
TSBY	226.61	0.378
WCB	164.75	0.275
WCB*	140.29	0.234

Table 6.3.1 TSBY: Trypticase Soya Broth supplemented with yeast extract; WCB: Wilkins Chalgren Broth; WCB*: Ultrafiltrated Wilkins Chalgren Broth.

6.3.1.2 SDS-PAGE analysis of the protein preparations obtained by ammonium sulphate precipitation of *S. sanguis* culture supernatants

Samples of the proteins precipitated from the supernatants using ammonium sulphate were analysed by SDS PAGE, the results of which are shown in figure 6.1. Although WCB* had proteins >10 KDa removed by ultrafiltration, the resulting bacterial supernatant (Fig. 6.1a, lane 7) contained some of the same bands as those found in WCB bacterial supernatant (Fig. 6.1a, lane 3). These proteins are therefore unlikely to be from the medium and are bacterial proteins. The control for WCB (Fig. 6.1a, lane 2) contained a large amount of material which was not present in the filtered WCB* control (Fig. 6.1a, lane 6). WCB* would therefore have been a good choice for large scale growth of *S. sanguis*. The number of protein bands seen in the bacterial supernatant grown in this medium was surprisingly low, while growth in TSBY (Fig.

6.1a, lane 5) produced a large number of proteins. Assessment of the supernatants' biological activity was also needed to decide on the medium of choice.

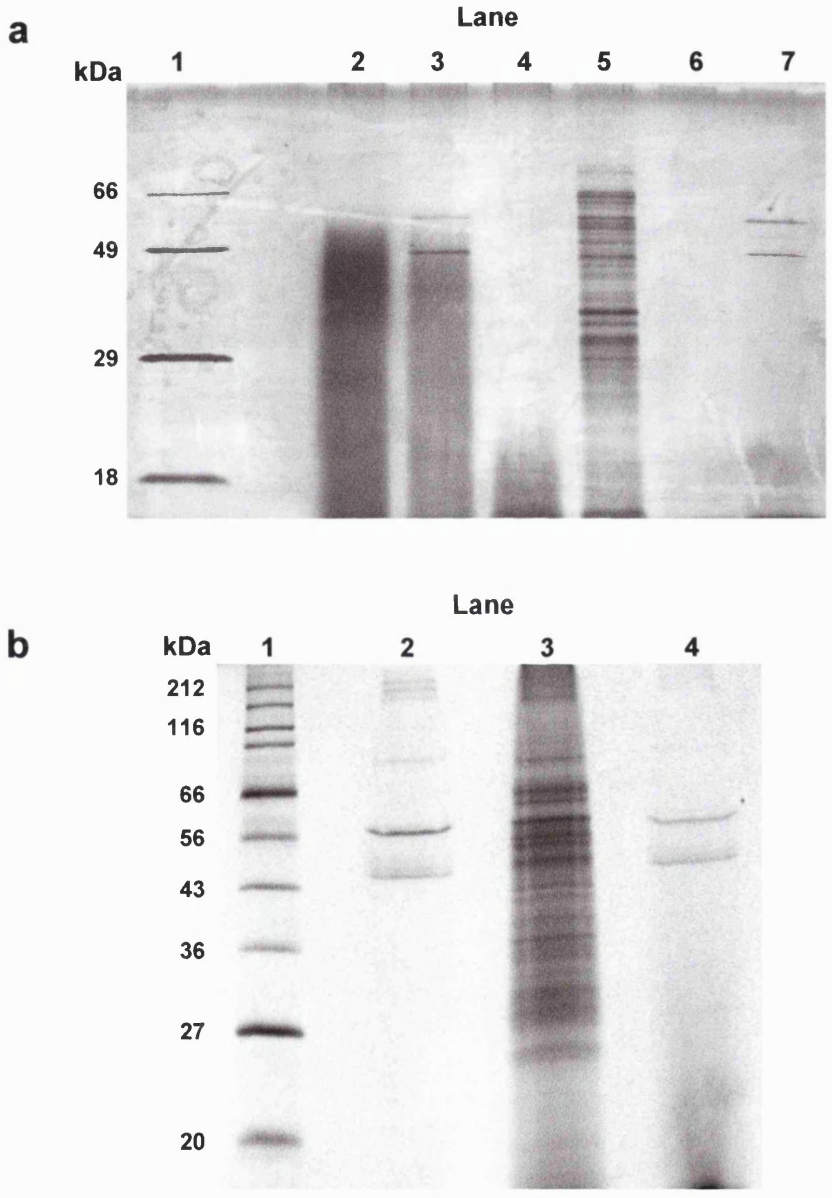

Figure 6.1 SDS-PAGE analysis of the protein preparations obtained by ammonium sulphate precipitation of bacterial supernatants and controls (without bacteria). The gel shown in panel (a) was visualised with silver stain. Lane 1 shows the molecular mass markers; lane 2 shows the WCB control; lane 3 shows the WCB-produced bacterial supernatant; lane 4 shows the TSBY control; lane 5 shows the TSBY-produced bacterial supernatant; lane 6 shows the WCB* control; lane 7 shows the WCB*-produced bacterial supernatant. The gel shown in panel (b) was stained with Colloidal blue. Lane 1 shows the molecular mass markers; lane 2 shows the WCB*-produced bacterial supernatant; lane 3 shows the TSBY-produced bacterial supernatant; lane 4 shows the WCB-produced bacterial supernatant. Controls were not examined by colloidal blue stain.

6.3.1.3 Cytokine-inducing activity of the protein preparations obtained by ammonium sulphate precipitation of *S. sanguis* culture supernatants

The ammonium sulphate-precipitated protein from all three bacterial supernatants stimulated PBMC to secrete IL-6 in a dose-dependent manner (figure 6.2). The protein from the WCB-produced bacterial supernatant induced the highest level of secreted IL-6 from PBMC, followed by the TSBY, and finally the WCB*-produced bacterial supernatant. WCB- and WCB*-produced supernatants had similar potency, inducing IL-6 at as little as 1μl/ml, while TSBY-produced supernatant was approximately ten times as potent, being active at between 1 and 0.1μl/ml.

To determine which of the three bacterial supernatants was most active, relative to its control, each was tested at a single concentration for its ability to induce IL-6 release from PBMC. Figure 6.3 shows that the control supernatants for WCB and WCB* induced PBMC to secrete IL-6, and in fact there was no significant difference between the effect of the bacterial supernatant produced in WCB* and its control ($P>0.05$). The biggest difference in activity was seen between the supernatant produced in TSBY and its control, the latter inducing a level of IL-6 secretion which was not significantly different from the unstimulated control cells ($P>0.05$).

Of the three types of growth medium tested, the TSBY bacterial supernatant contained the largest number of proteins as revealed by silver-stained SDS-PAGE, and was the most potent in inducing IL-6 secretion from PBMC. The TSBY control preparation was inactive in the PBMC assay and contributed relatively few proteins to the bacterial supernatant, as revealed by silver-stained SDS-PAGE. Together these results led to the decision to scale up the growth of *S. sanguis* in TSBY.

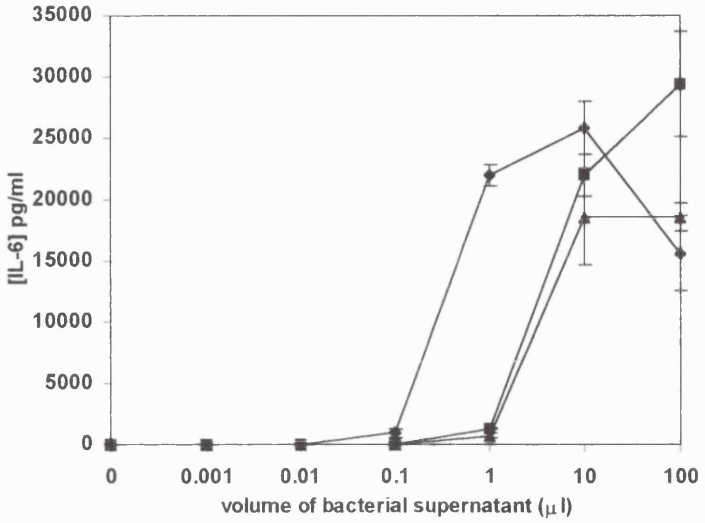

Figure 6.2 Effect, on IL-6 production by PBMC, of various volumes of protein preparations from *S. sanguis* culture supernatants produced by growth in TSBY (diamond), WCB (square) or WCB* (triangle). The results are expressed as mean and SD of three replicate cultures.

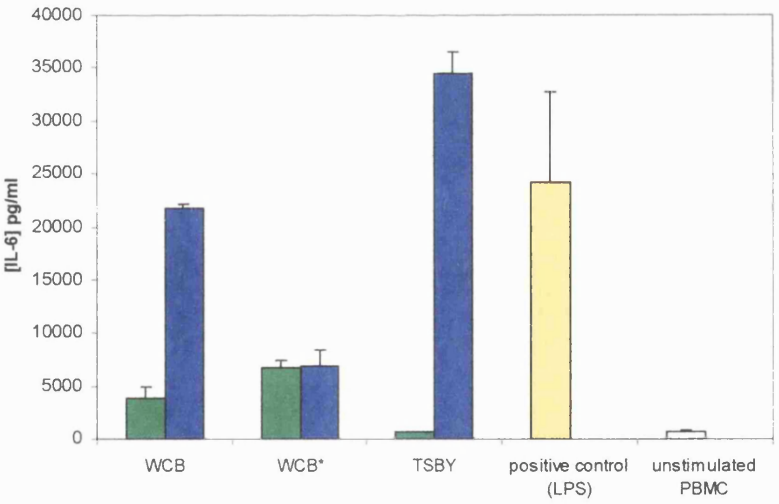

Figure 6.3 IL-6-inducing activity of the protein preparations from *S. sanguis* culture supernatants produced using different media (blue bars), and their respective controls (green bars). In each case 0.3µl/ml of the protein preparation from the culture supernatant was tested. The positive control represents PBMC cultures stimulated with 10 ng/ml *E. coli* LPS (yellow bar), and the negative control represents unstimulated PBMC cultures (white bar). The results are expressed as the mean and SD of triplicate cultures.

6.3.2 Scale-up of *S. sanguis* supernatant production

6.3.2.1 Protein content and SDS-PAGE analysis

The protein content of the dialysed ammonium sulphate precipitated proteins was 4mg/ml for each of the two scaled-up batches of *S. sanguis* grown in TSBY. To assess the reproducibility of different batches of *S. sanguis* supernatant as far as protein profiles were concerned, samples were separated on 12% SDS-PAGE gels, silver stained, and the protein profiles compared. Figure 6.4 shows the variation in the protein profiles of the two batches of supernatant. The main differences were in proteins greater than 66kDa in size.

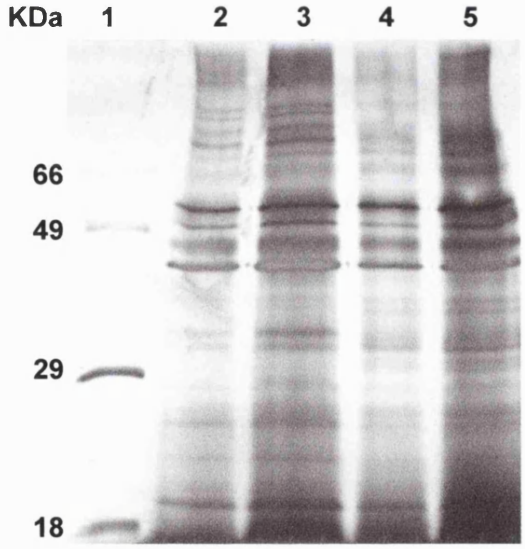

Figure 6.4 SDS-PAGE analysis of protein from two different batches of *S. sanguis* culture supernatants produced in TSBY. 15µl and 36µl of each supernatant was separated on a 12% gel and stained with silver stain. Lane 1 shows the molecular weight markers. Lanes 2 and 3 show one batch of protein (15 and 36µl respectively); Lanes 4 and 5 show a second batch of protein (15 and 36µl respectively).

6.3.2.2 Cytokine-inducing activity of large scale *S. sanguis* culture supernatant protein preparations

The protein preparation stimulated PBMC to secrete IL-6 at >0.4mg/ml protein in a dose-dependent fashion, with levels of IL-6 continuing to increase up to 40mg/ml protein, after which the level of IL-6 decreased (figure 6.5a). The ability to induce IL-6 secretion in PBMC was completely abolished by heating the protein in a boiling water-bath for 30 minutes, before testing in the PBMC assay (figure 6.5b).

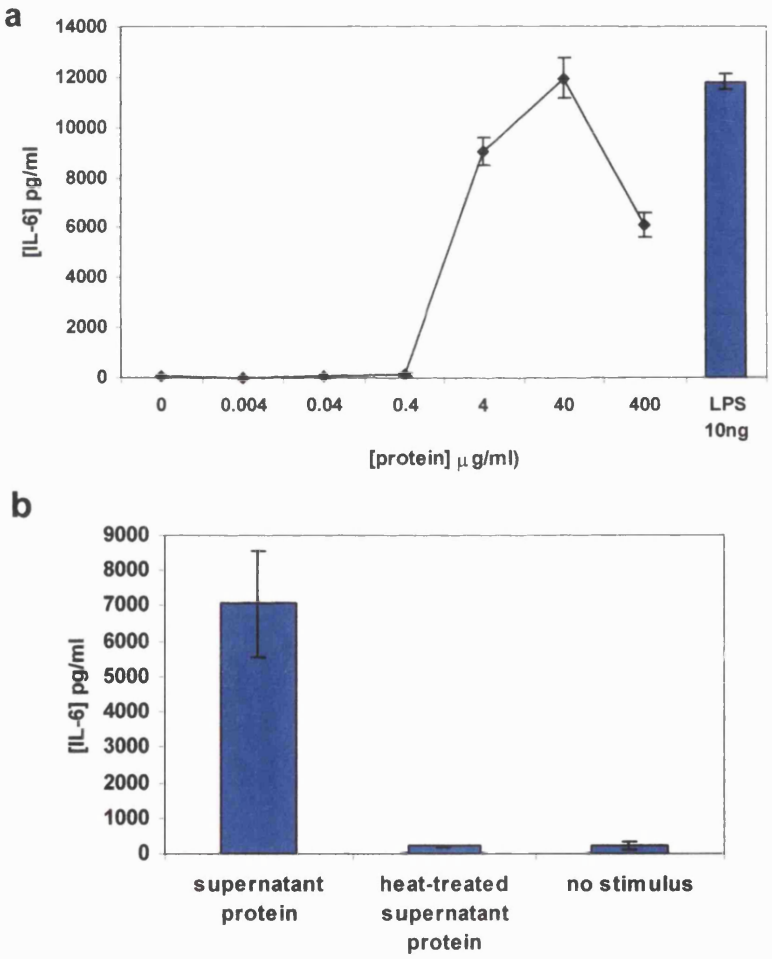

Figure 6.5 Cytokine-inducing activity of the large scale protein preparation from *S. sanguis* culture supernatant tested on PBMC. Panel (a) shows IL-6 secretion by PBMC in response to various amounts of *S. sanguis* protein. *E. coli* LPS at 10 ng/ml was tested as a positive control. Panel (b) shows the effect of heat treatment (30 minutes in a boiling water bath) on the cytokine-inducing activity of the same protein preparation. Supernatant protein was tested at 1μg/ml. Results are expressed as the mean and SD of triplicate cultures.

6.3.2.3 Fractionation of protein from *S. sanguis* culture supernatants: trial run using an Econopac Q anion exchange cartridge

Almost all the protein in the preparation from *S. sanguis* culture supernatant bound to the anion exchange cartridge, as shown by the absence of bands in the run-off collected during loading of the sample (run-off I) and that collected during the subsequent washing step (run-off II), (figure 6.6a lanes 3 and 4). The addition of buffer B containing 1M NaCl released all of the bound protein from the cartridge, the majority of which eluted in two fractions (lanes 6 and 7). The resulting fractions were assessed for IL-6-inducing activity in the standard PBMC assay (figure 6.6b). The majority of the activity was recovered in fraction 7, corresponding to the peak of protein elution. This activity was equal to that of whole, un-fractionated supernatant, confirming that activity was not lost during separation on the Bio-Rad Econopac Q cartridge, and thus confirming that the matrix used in these columns would be suitable for subsequent larger scale separations.

6.3.2.4 Fractionation of protein from *S. sanguis* culture supernatants: trial run using an Econopac S cation exchange cartridge

As for the Econopac Q cartridge, all fractions were separated on 12% SDS-PAGE gels and visualised by silver stain, as well as being tested in the routine PBMC assay for IL-6-inducing activity. Not all of the protein present in the supernatant bound to the Econopac S cartridge; both the run-off collected during the loading of sample (run-off I) and that collected during the subsequent wash with buffer A to remove un-bound protein (run-off II), contained numerous protein bands, (figure 6.7a, lanes 3 and 4 respectively). A large amount of activity was eluted in the first run-off, (figure 6.7b, sample 3). On addition of buffer B containing 1M NaCl a large number of proteins were recovered (figure 6.7a, lanes 6 and 7). However, no activity was detected in these fractions (figure 6.7b, samples 6 and 7), suggesting that either these proteins were inactive, or that they had lost activity as a result of being passed down the cation exchange chromatography cartridge. Interestingly, once the cartridge was returned to buffer A a second peak of protein was eluted (figure 6.7a, lanes 10 to 16). Substantial

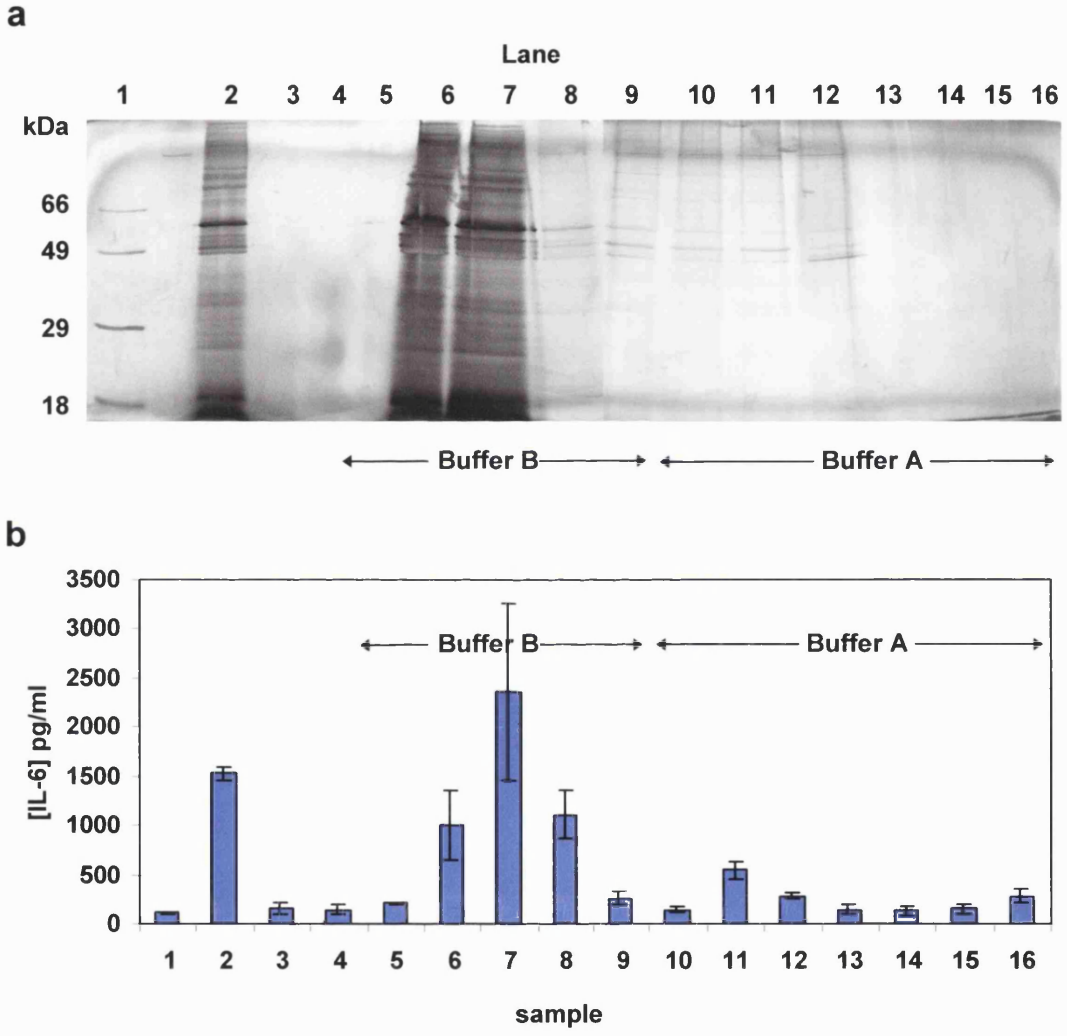

Figure 6.6 Fractionation of protein from *S. sanguis* culture supernatant using an Econopac Q anion exchange chromatography cartridge. Panel (a) shows the fractions run on 12% SDS PAGE gels and silver stained. Lane 1 shows the molecular mass markers; lane 2 shows the protein before separation; lane 3 shows run-off I; lane 4 shows run-off II; lanes 5-9 show the fractions collected during elution with 1M NaCl; lanes 10-16 show fractions collected once the buffer was changed back to equilibration buffer. Panel (b) shows the IL-6-inducing activity of the fractions, as assessed using the routine PBMC assay. Sample 1 represents the negative control of elution buffer only; all other sample numbers correspond to the lane numbers in panel (a). Results are expressed as the mean and SD of triplicate cultures.

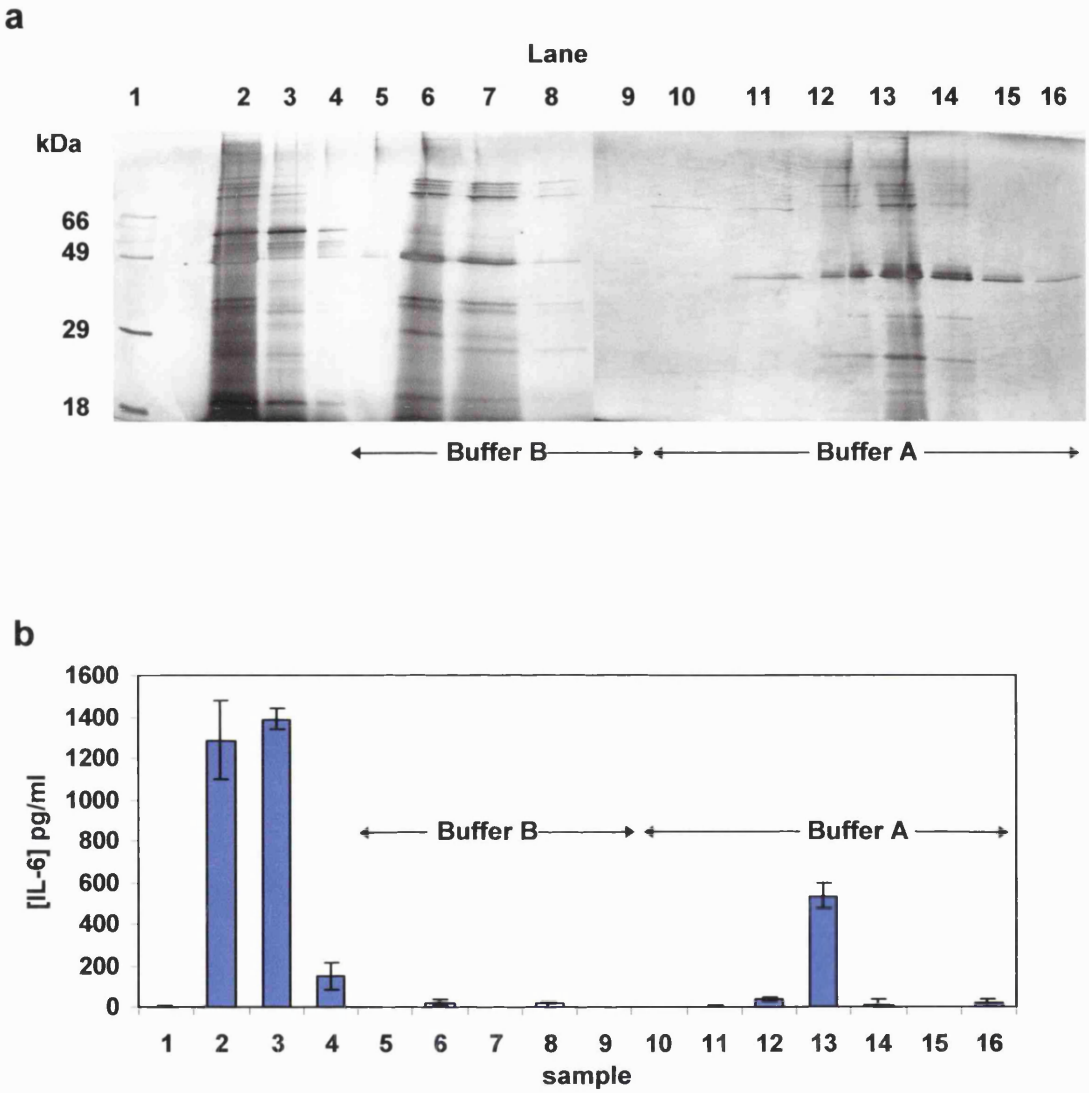

Figure 6.7 Fractionation of protein from *S. sanguis* culture supernatant using an Econopac S cation exchange chromatography cartridge. Panel (a) shows the fractions run on 12% SDS PAGE gels and silver stained. Lane 1 shows the molecular mass markers; lane 2 shows the protein before separation; lane 3 shows run-off I; lane 4 shows run-off II; lanes 5-9 show the fractions collected during elution with 1M NaCl; lanes 10-16 show fractions collected once the buffer was changed back to equilibration buffer. Panel (b) shows the IL-6-inducing activity of the fractions, as assessed using the routine PBMC assay. Sample 1 represents the negative control of elution buffer only; all other sample numbers correspond to the lane numbers in panel (a). Results are expressed as the mean and SD of triplicate cultures.

Both anion and cation exchange chromatography looked promising as methods for isolation of individual proteins from the *S. sanguis* supernatant. Anion exchange chromatography didn't actually fractionate any of the proteins as everything bound to the cartridge, but with elution performed with a sodium chloride gradient, rather than as a step-wise addition, this method would probably fractionate proteins quite successfully. Since cation exchange chromatography fractionated the proteins into three portions (unbound proteins, proteins eluted with 1M NaCl buffer (buffer B) and a second elution peak on return to buffer A) in just one purification step, it was decided to proceed with large scale cation exchange chromatography in the first instance.

6.3.2.5 Fractionation of the protein from *S. sanguis* culture supernatant using an S20 cation exchange HPLC column.

To aid the reader, a flow diagram has been included so that the discussion of the purification schemes described in the remainder of this chapter can be followed more easily (Figure 6.8).

Figure 6.9 shows the SDS-PAGE analysis of the fractions generated by S20 cation exchange chromatography (using the batch elution method) of ammonium sulphate precipitated protein from *S. sanguis* culture supernatant. Proteins were run on 12% gels and visualised with silver stain. The run-off and wash (containing unbound material) contained a large number of bands. The former retained substantial IL-6-inducing activity and so was kept for further purification at a later stage, (figure 6.10, sample 3). The fractions which were collected once the buffer had been changed back from B to A contained a peak of IL-6-inducing activity centering around fraction 26, (figure 6.10, samples 25-27). Although these active fractions contained numerous protein bands, the predominant protein of approximately 45kDa was present in the largest amount in fraction 26, and thus corresponded with the active peak. It seemed likely that this protein was responsible for the IL-6-inducing activity of fractions 25-27, and since all other proteins in these fractions were only present in very small amounts, purification of the 45kDa protein was already well underway.

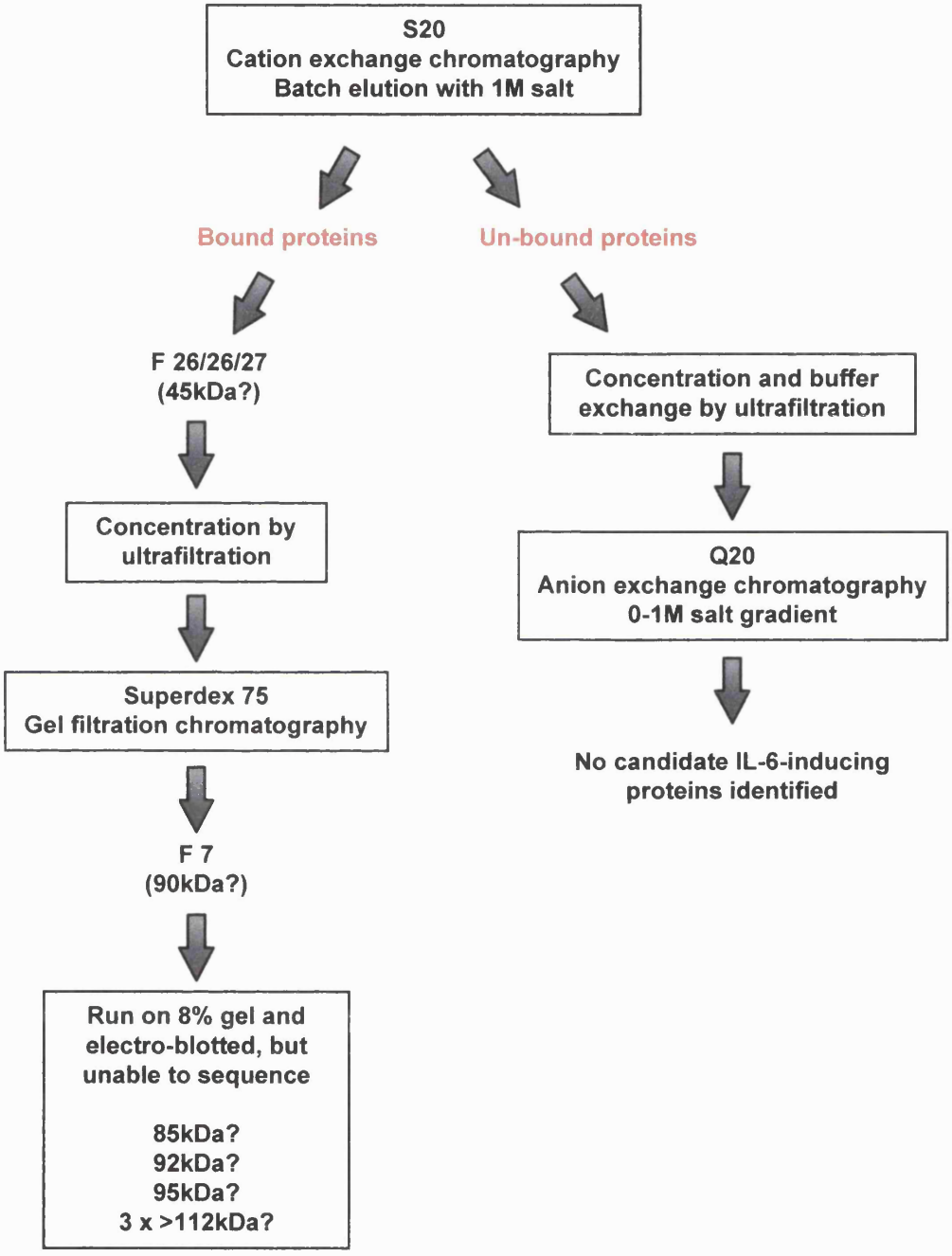

Figure 6.8 Flow diagram of the isolation of IL-6-inducing proteins from *S. sanguis* culture supernatant protein. Purification and N-terminal sequencing steps are represented by rectangular boxes. Fractions are denoted by F followed by the fraction number. At each stage the molecular mass of the putative IL-6-inducing proteins is given in brackets beneath the appropriate fraction. The question mark indicates the protein(s) thought most likely to be the cytokine-inducing components at that stage in the purification.

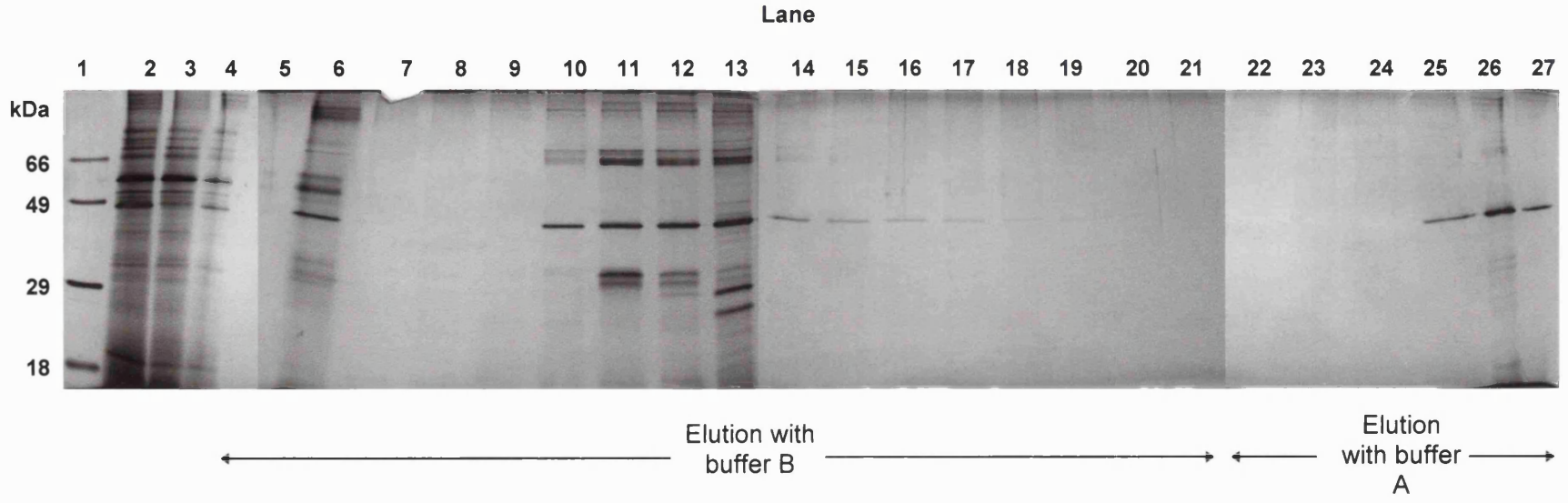

Figure 6.9 Fractionation of proteins from *S. sanguis* culture supernatant using an S20 cation exchange HPLC column. The fractions obtained were run on 12% SDS PAGE gels and silver stained. Lane 1 shows the molecular weight markers; lane 2 shows the protein before separation; lanes 3 and 4 show run-off I and II respectively; lanes 5 to 21 show the fractions collected during elution with buffer B (each fraction comprising three pooled 4ml fractions); lanes 22 to 27 show the first 6 fractions collected after the buffer was returned to buffer A (these are not pooled fractions but individual 4 ml fractions). The remaining fractions did not contain any protein bands visible with silver stain and are therefore not shown here.

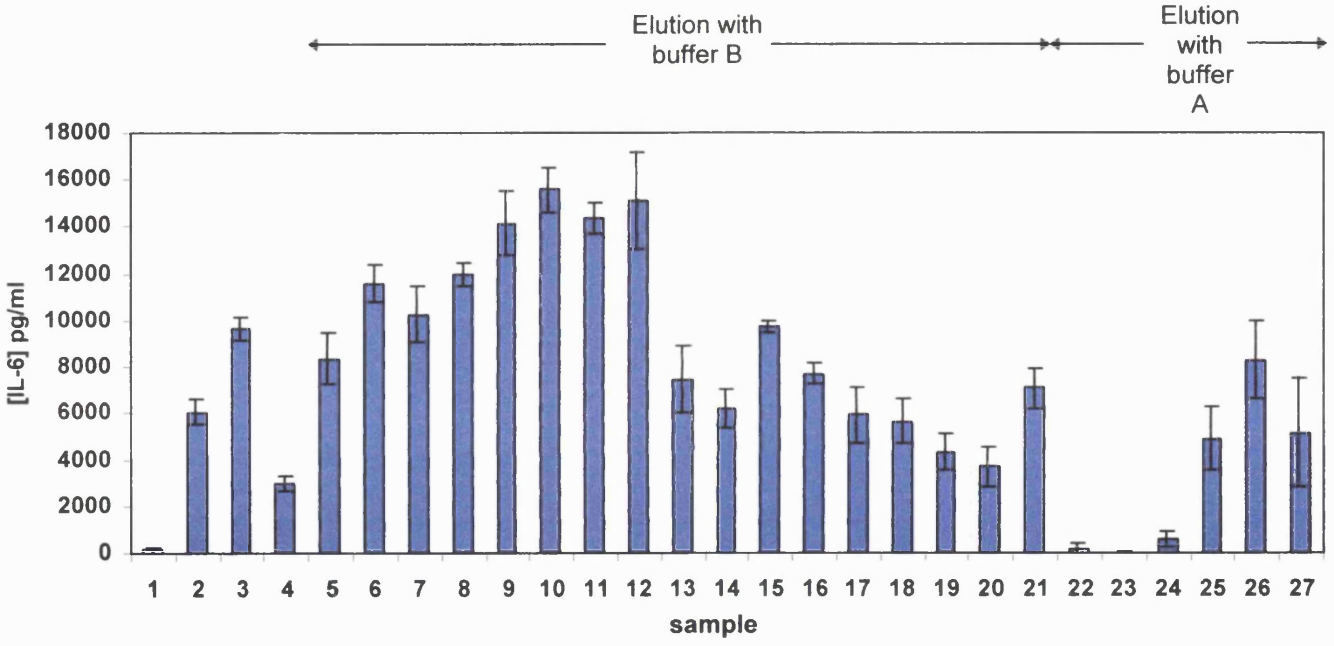

Figure 6.10 Fractionation of protein from *S. sanguis* culture supernatant using an S20 cation exchange HPLC column: IL-6-inducing activity. Sample 1 represents the negative control of buffer B only; sample 2 is the protein before fractionation; samples 3 and 4 are run-off I and II respectively; samples 5 to 21 are the fractions collected during elution with buffer B (each fraction comprising three pooled 4ml fractions); samples 22 to 27 are the first 6 fractions collected after the buffer was changed back to buffer A (these are not pooled fractions but individual 4 ml fractions). Apart from sample 1, all sample numbers correspond to the lane numbers in figure 6.9. The remaining fractions had no IL-6-inducing activity and are not shown here. Results are expressed as the mean and SD of triplicate cultures.

The remaining 120ml of sample was fractionated on the S20 column as described previously. To confirm that the S20 column had not been overloaded with protein, run-off containing unbound protein was re-loaded and eluted following the same method as for the actual fractionation. Silver-stained gels revealed that there was very little of the 45kDa protein present in the resultant fractions, confirming that the column had bound almost all of this protein and had not been overloaded (results not shown). To purify the 45kDa protein further, all fractions containing this band were pooled, concentrated by ultrafiltration and then separated by gel filtration chromatography.

6.3.2.6 Gel filtration chromatography of fractions 25-27

The protein eluted from the gel filtration column in two main peaks (figure 6.11). The first, Peak 1, eluted in the void volume of the column with the majority of the protein being collected in fraction 7. The second, Peak 2, was a much broader peak, possibly comprising several smaller merged peaks of protein. The proteins from Peak 2 were collected in fractions 9 to 25, with the highest point of the protein peak corresponding to fractions 13 to 15. All fractions were tested for IL-6-inducing activity and the activity corresponded to Peak 1 of eluted protein (figure 6.11). No other fractions were found to be active in this assay. This was a slightly unexpected result since the activity had been thought to be due to the 45kDa protein and a protein of this mass should not have eluted in the column void volume: the exclusion limit of the Superdex 75 column is approximately 90kDa, and anything larger than this elutes in the void volume. However, if the 45kDa protein had existed as a multimer in its native form, it could elute in the void volume, since even a dimer would be about 90kDa in size. On a reducing gel such a multimer would be seen in its monomeric form.

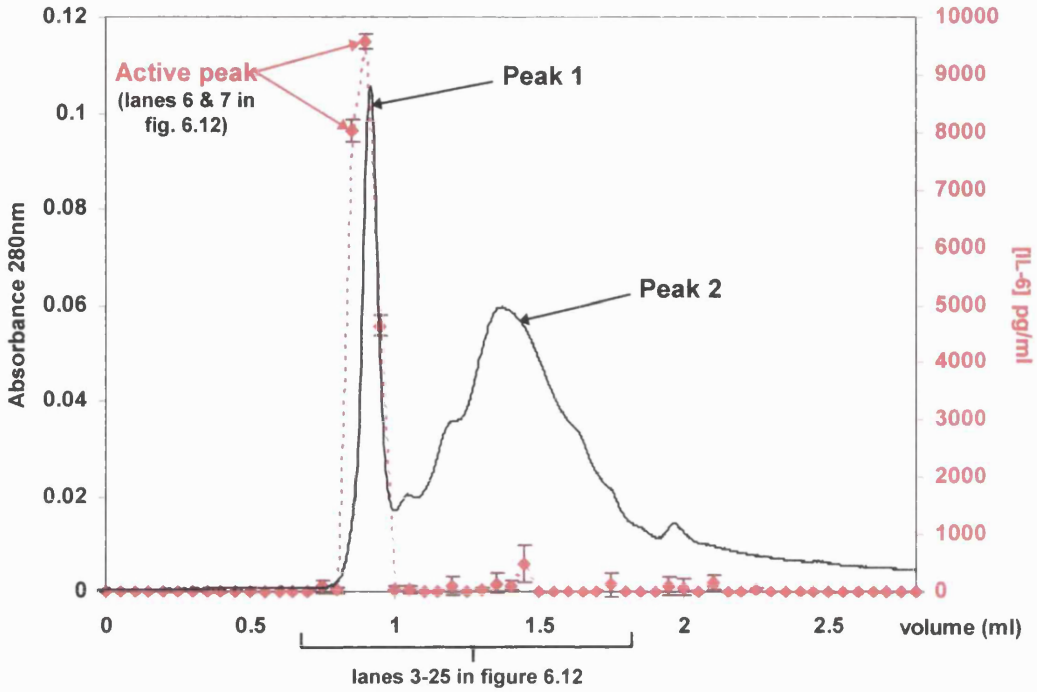

Figure 6.11 Superdex 75 gel filtration chromatography of fractions 25-27 from the previous cation exchange chromatography step. The column was equilibrated with phosphate buffered saline (PBS). The protein elution profile is shown as absorbance at 280nm (solid black line). Each fraction was assayed for IL-6-inducing activity (dotted red line).

Examination of the proteins present in the active fraction by SDS-PAGE revealed several candidates for this activity (figure 6.12, lane 7). The fraction contained several high molecular mass proteins, the predominant protein being a doublet of approximately 90kDa. Interestingly the 45kDa eluted in fraction 10, a fraction with little activity. Since the 45kDa protein did not correspond to IL-6-inducing activity (which was recovered in peak 1) these results suggested that the IL-6-inducing activity was not due to the 45kDa protein.

The main candidate proteins for the IL-6-inducing activity were of high molecular mass and so N-terminal sequencing would require quite large amounts. Since further purification steps would undoubtedly result in more loss of protein, it was decided to proceed to N-terminal sequencing of as many of the protein bands in the IL-6-inducing fraction as possible.

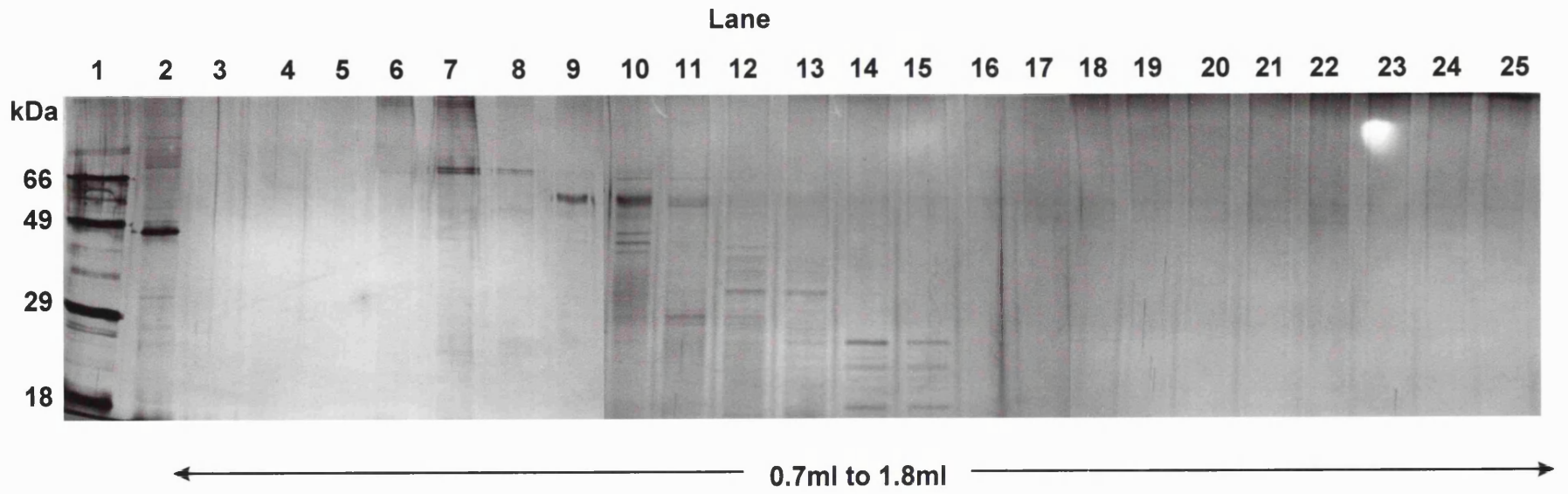

Figure 6.12 Superdex 75 gel filtration chromatography of fractions 25-27 from the previous cation exchange chromatography step. Fractions corresponding to the main peaks of absorbance at 280nm (eluted between 0.7 and 1.8ml during the run, and shown by the double-headed arrow in figure 6.11) were separated on 12% gels and silver stained. Lane 1 shows the molecular mass markers; lane 2 shows fraction 26 before fractionation by gel filtration chromatography; lanes 3 - 25 show the fractions eluted between 0.7 and 1.8ml, as indicated by the double-headed arrow in figure 6.11. Fractions collected after 1.8ml showed no major peaks of absorbance at 280nm and contained no visible protein bands when analysed by silver-stained SDS-PAGE, and are therefore not shown here.

6.3.2.7 SDS-PAGE and electro-blotting of fraction 7 for N-terminal sequencing.

All the molecular mass markers were clearly visible on the blot after staining. Unfortunately no bands were visible in either of the two sample lanes. The gel itself was then stained with coomassie blue to see if the proteins had failed to transfer during the blotting. Several bands were visible in the sample lanes of the gel (figure 6.13). Separation of the fraction on this 8% gel allowed for a more accurate estimation of the molecular masses of the candidate active proteins than had been possible on the 12% gel (figure 6.12). A standard curve of molecular mass standards was used as described in Chapter 2, section 2.3.5. Three proteins of greater than 212kDa (visible in figure 6.13) and three much less strongly-stained proteins of 85, 92 and 95 kDa were present in the active fraction, and any of these could be responsible for the induction of IL-6 secretion from PBMC in the bioassay. The bands of around 90kDa which had been the predominant proteins in fraction 7, were much feinter than the other higher molecular mass proteins. This suggested that some of the protein had been transferred successfully to the PVDF membrane, but that this was insufficient protein to be visible after just 10 minutes in stain, and therefore insufficient protein to obtain N-terminal sequence data from.

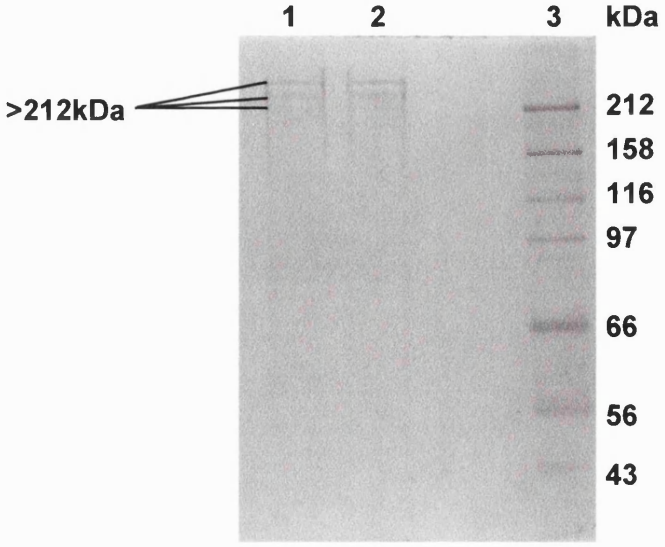

Figure 6.13 SDS-PAGE analysis of the active fraction (fraction 7; Peak 1 in figure 6.11) recovered from gel filtration chromatography of fractions 25-27. The photograph shows the active fraction separated on an 8% gel stained with coomassie blue. Lanes 1 and 2 show the active fraction; lane 3 shows the molecular mass markers.

6.3.3 SDS-PAGE analysis of whole, unfractionated *S. sanguis* culture supernatant protein, and the S20 column run-off protein

Figure 6.14 shows the protein profile of the material that didn't bind to the S20 HPLC column ("run-off") compared to the original ammonium sulphate precipitated protein before cation exchange chromatography. A large number of proteins did not bind to the S20 cation exchange column. However, some of the proteins of higher molecular mass were less abundant in the run-off, as was a protein of approximately 48 kDa in size. Since the run-off had previously been shown to have retained substantial IL-6-inducing activity (figure 6.10, sample 3), the protein was further fractionated by anion exchange chromatography using a Q20 HPLC column.

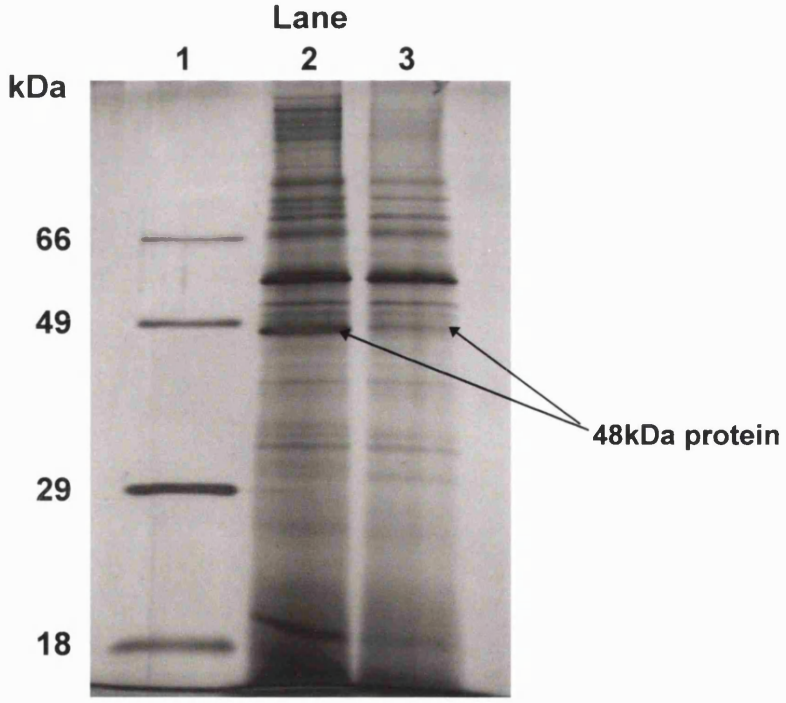

Figure 6.14 SDS-PAGE analysis of protein obtained by ammonium sulphate precipitation of *S. sanguis* culture supernatant, and run-off (unbound material) collected during fractionation using an S20 cation exchange HPLC column. Lane 1 shows the molecular mass markers; lane 2 shows the original protein before cation exchange chromatography; lane 3 shows the unbound protein collected during the application of protein onto the S20 column.

6.3.3.1 Anion exchange chromatography using a Q20 HPLC column

The elution profile for the Q20 fractionation showed no clearly defined peaks of protein as all the values were off the absorbance scale (chromatogram not shown). However, SDS-PAGE analysis revealed that a good separation had been achieved (figure 6.15a). Fractions 5, 6, 7 and 8 all contained numerous bands, although in each there was at least one protein that had eluted almost exclusively in that fraction. For example, amongst the other protein bands, fraction 6 contained one densely-stained band of about 55 kDa that had eluted predominantly in this fraction, as well as bands of about 35 and 20kDa which didn't appear to be present in neighbouring fractions. The majority of the high molecular mass proteins eluted at a fairly low salt concentration (<0.2M NaCl) and were present mainly in fractions 4,5 and 6. Thus far, the fractionation of the proteins looked promising. However, IL-6-inducing activity was centred around fractions 11 to 14, with lower activity in fractions 8, 9 and 10 (figure 6.15b). Looking at these results together with the SDS-PAGE analysis, there were no obvious candidate proteins for this activity: all the bands seen in the most active fractions were also present in fractions with much lower IL-6-inducing activity. Perhaps more importantly, any proteins that might have been active were present in very small amounts. As there were no clear leads, it was decided not to proceed further with this particular purification.

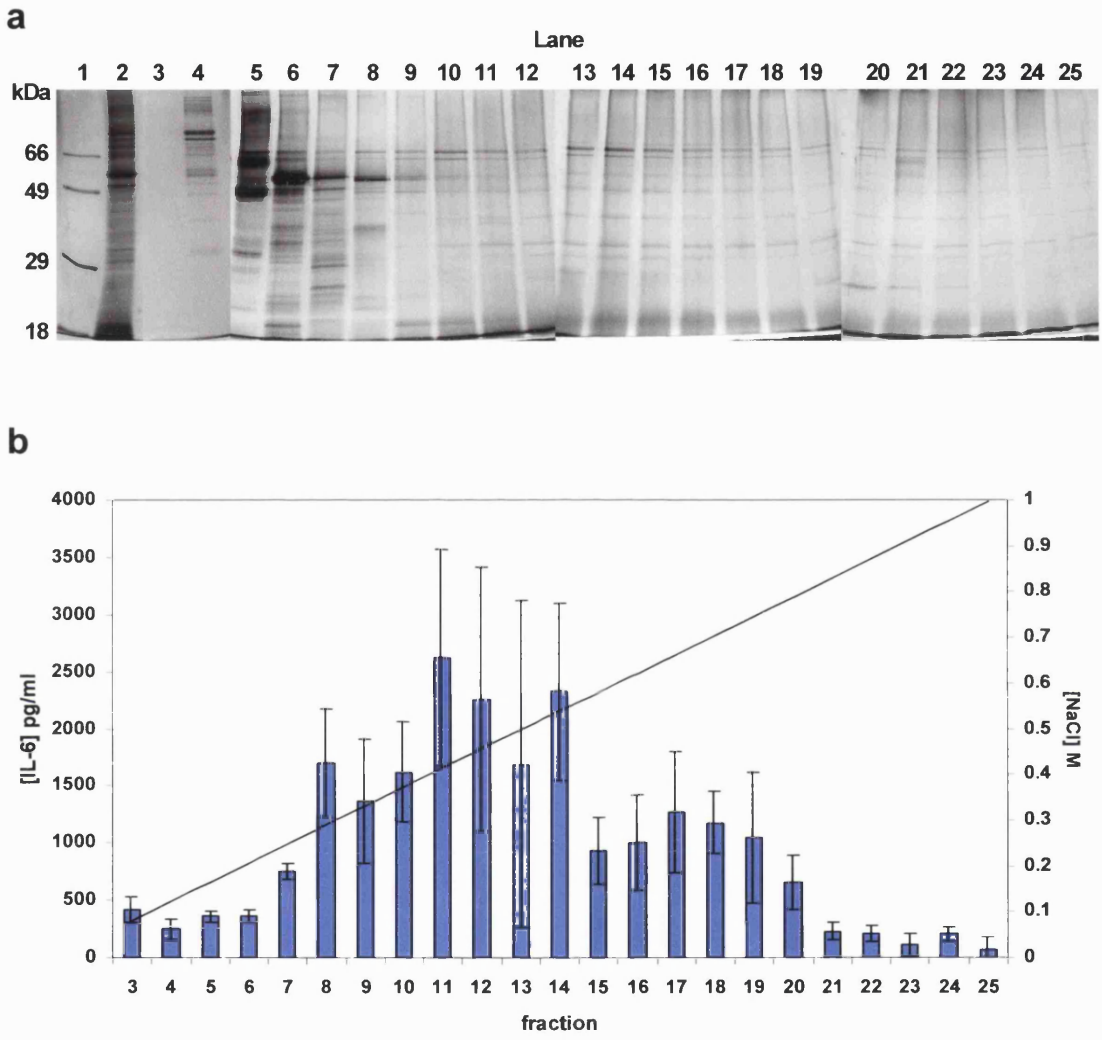

Figure 6.15 Q20 anion exchange HPLC fractionation of unbound protein ("run-off") from the previous HPLC cation exchange chromatography using an S20 column. Panel (a) shows the SDS-PAGE analysis of the fractions. Lane 1 shows the molecular mass markers; lane 2 shows the unfractionated "run-off"; lanes 3-25 show the fractions eluted over a 0-1M NaCl gradient. Panel (b) shows the IL-6-inducing activity of the same fractions, as determined in the routine PBMC assay. The fraction numbers correspond to the lane numbers in panel (a). The NaCl gradient is represented by the solid black line. Data are represented as the mean and standard deviation of triplicate samples.

6.4 Discussion

In 1992 Bayston *et al* published the first systematic study of the ability of cell-free supernatants of common Gram-positive bacteria to induce TNFα secretion from whole human blood (Bayston *et al.* 1992). Of the 63 strains tested, all induced TNFα secretion but, interestingly, the organisms generally considered to be of low virulence, including *S. sanguis*, induced levels of secreted TNFα comparable to those induced by more virulent organisms such as *Staph. aureus*.

A later study focusing on the oral streptococci found that cell-free supernatants from *S. sanguis* induced the secretion of TNFα, TNFβ and IL-8 from isolated human PBMC (Soto *et al.* 1996). Another study by the same group reported that supernatants from clinical isolates of viridans streptococci induced significantly more TNFβ and IL-8 than did supernatants from commensal strains (Soto *et al.* 1998). In view of the role of IL-8 in the pathogenesis of the acute respiratory distress syndrome (ARDS), one of the principal clinical features of the alpha-streptococcal shock syndrome, it has been suggested that the secreted products from viridans streptococci may be important in the pathogenesis of this disease.

None of the above studies attempted to isolate the individual components responsible for the cytokine-inducing activity. In 1993 Takada *et al* published a report describing an extracellular cytokine-inducing factor from viridans streptococci (*S. mitis* and *S. oralis*) (Takada *et al.* 1993). Various chromatography techniques were used to purify a proteinaceous fraction that induced serum IL-6, TNFα and IFNγ when injected intravenously into mice. The fraction also induced the secretion of IL-6, TNFα and thymocyte-activating factor (IL-1β) in isolated human monocytes, whole blood and murine peritoneal macrophages. Although the protein(s) responsible for this activity were not purified to homogeneity in this study, the active fraction was composed of proteins greater than 150kDa.

The aim of the study described in this chapter was to isolate the individual components from cell-free culture supernatants of *S. sanguis* that were responsible for inducing cytokine production by human PBMC.

Three types of liquid medium were investigated for their suitability for the production of secreted protein from planktonically-grown *S. sanguis*. Wilkins Chalgren agar had previously been used for the production of surface-associated material as detailed in Chapter 3, and *S. sanguis* NCTC 10904 grew well on this solid medium when supplemented with 5% horse blood. Without the blood supplement this strain grew less vigorously, but the addition of blood to culture broth would lead to the inevitable problem of how to remove the blood components from the culture supernatants containing bacterial secreted protein. So a comparison was made of planktonic growth in Wilkins Chalgren broth with growth in Trypticase soya broth supplemented with yeast extract (often the medium of choice for the growth of streptococci).

In procedures for isolating and purifying bacterial proteins, there is always the possibility of contamination by constituents of the growth medium in bacterial preparations. To reduce this problem, a separate batch of Wilkins Chalgren broth was subjected to ultrafiltration to remove molecules greater than 10kDa, before autoclaving. Trypticase soya broth consists of enzymatically digested casein and soybean meal and contains only low molecular mass proteins or peptides. Most bacterial proteins secreted into TSBY would therefore be easily distinguished from medium-derived molecules by their size, and so ultrafiltration of this medium was deemed unnecessary.

The three types of medium were compared for their effects on bacterial yield, production of secreted protein, and for whether the medium components themselves stimulated isolated PBMCs to produce cytokines. TSBY was the most promising candidate: it produced the highest yield of bacteria, and by far the largest number of proteins in the culture supernatant. More importantly, TSBY itself did not induce cytokine production by human PBMC in the routine assay which would be used later on as the screening procedure for identifying cytokine-inducing bacterial proteins.

S. sanguis culture supernatant protein was therefore produced on a larger scale in TSBY broth. This protein was demonstrated to induce PBMC to secrete IL-6, and the cytokine-inducing activity shown to be heat-labile, confirming that the activity was due to the protein and not to contaminating molecules such as LTA.

Initial experiments to find a suitable ion exchange chromatography matrix for fractionation of *S. sanguis* culture supernatant proteins were carried out using Bio-Rad ion exchange chromatography cartridges. A simple batch method of fractionation (i.e. a method that didn't use a salt gradient for elution of bound proteins) showed that IL-6-inducing activity was recovered successfully after fractionation using either the cation or the anion exchange chromatography cartridge. Large scale HPLC columns, packed with the same matrices as the cartridges, were thus employed for initial fractionation of the culture supernatant protein.

The batch method of fractionating protein using the Bio-Rad Econopac S cation exchange chromatography cartridge had revealed an interesting, and potentially useful, phenomenon. Although a large proportion of bound protein eluted almost immediately on addition of elution buffer (1M NaCl in 20mM MOPS pH 6.0), this did not exhibit IL-6-inducing activity. The only IL-6-inducing activity found in the proteins that had bound to the cation exchange matrix, was present in a second peak of protein that eluted as the cartridge was returned to equilibration buffer containing no NaCl. This phenomenon was reproducible, and was not due to insufficient volume of elution buffer being passed through the cartridge, as SDS-PAGE analysis revealed that the last two fractions collected during NaCl elution contained no protein bands. It is possible that the addition of 1M NaCl may have changed the proteins in some way, perhaps inducing conformational changes that altered their binding characteristics. If ionic interactions alone were involved in the binding of these proteins to the matrix, then it seems unlikely that a buffer containing no NaCl would be more able to disrupt binding than a buffer containing 1M NaCl, especially since it allowed binding to occur in the first instance. This suggests that other interactions may be involved. Whatever the reasons, this phenomenon allowed the separation of IL-6-inducing proteins from a large number

of inactive proteins in a single step, and so this method was employed on a larger scale to fractionate the remaining ammonium sulphate-precipitated protein from the *S. sanguis* culture supernatant.

Fractionation by cation exchange chromatography using a Bio-Rad S20 HPLC column with batch elution as used with the cartridges, led to the separation of two main groups of proteins. As had been observed for the Econopac S cartridge, a large number of proteins eluted as soon as the 1M NaCl elution buffer was applied. However, using this column, the proteins that eluted with 1M NaCl were active in inducing IL-6 secretion from human PBMC. Since the matrix used in the Econopac S cartridge is the same as that used in the S20 HPLC column, it is unlikely that the matrix had affected the activity of the proteins eluted from the cartridge. It may be that if a smaller volume of each fraction had been tested in the routine PBMC assay we might have observed a result closer to that seen with the fractions from the cartridge separation, particularly if the activity of the second peak of proteins, which eluted on return to equilibration buffer, was substantially more active than the first.

Despite the finding that the proteins eluted by 1M NaCl induced IL-6 secretion from PBMC, these fractions contained a large number of proteins likely to require many further purification steps. In contrast, the second peak of protein also induced IL-6 secretion but contained only one protein present in large amounts, a protein of approximately 45 kDa. It was therefore decided to attempt to purify this protein by gel filtration chromatography to remove the other less abundant proteins from the active fraction.

On gel filtration chromatography using a Superdex 75 column only a small amount of 45 kDa protein was eluted. The loss of this protein could be due to several factors: the protein may have stuck to either the ultrafiltration spin-cell or the Microcon concentrator during concentration of the active fractions prior to gel filtration chromatography, or it might have bound to some component of the gel filtration column and not been eluted. The matrix is a composite of cross-linked agarose and dextran,

and the streptococci are known to produce several molecules that can bind dextran. Glucosyltransferases (GTFs) catalyse the synthesis of glucans, both water insoluble (e.g. mutans) and soluble (e.g. dextran), and GTFs have binding sites for glucan. *S. sobrinus* expresses a glucan-binding lectin that binds to high molecular weight glucans (Denson and Doyle, 1998), and *S. mutans* has been shown to express glucan-binding proteins, (Sato *et al.* 1997; Haas and Banas, 2000). Alternatively, the 45kDa protein may have degraded during some part of the procedure and lost its activity. However, IL-6-inducing activity was recovered in other gel filtration fractions, leading to the conclusion that the activity was not due to the 45 kDa protein.

The activity eluted in the void volume of the column, suggesting a protein or proteins of >100 kDa (the size exclusion limit of the Superdex 75 column), and the relevant fractions did indeed contain high molecular mass proteins as revealed by SDS-PAGE analysis. The predominant proteins were a pair of about 100 kDa, which, due to their large mass, would require a large amount of protein for N-terminal sequencing. For this reason it was decided to proceed straight to SDS-PAGE and electroblotting to avoid loss of protein during further purification steps. Unfortunately the proteins failed to transfer completely to the PVDF membrane during electroblotting, and there was not enough to obtain N-terminal sequence data for any of the proteins. However, by staining the gel, which contained a small amount of un-transferred protein, after electroblotting, it was possible to see all six of the proteins in the active fraction. Since the proteins had been separated on an 8% gel, the molecular masses could be more accurately calculated. They were estimated to be 85, 92, 95, and three proteins of 212 kDa or more in size.

Attempts to isolate sufficient amounts of the IL-6-inducing protein(s) from *S. sanguis* culture supernatants for N-terminal sequence analysis may continue, but will extend beyond the time-span available for this study. If more time were available, the purification protocol developed here would be applied to a much larger initial volume of culture supernatant, perhaps using chemostat culture for the growth of *S. sanguis*. The high molecular mass proteins purified by cation exchange chromatography may be

better separated by the use of a gel filtration column with a higher optimal separation range than the Superdex 75 column used in this study (3-75,000 daltons).

Attempts were also made to isolate the IL-6-inducing components from the bacterial proteins that hadn't bound to the S20 HPLC column during the initial cation exchange chromatography. The protein was fractionated by anion exchange chromatography using a Bio-Rad Q20 HPLC column, but the active fractions only contained very small amounts of protein as assessed by SDS-PAGE. With no obvious leads to pursue, purification was not taken further in this study.

CHAPTER 7

ISOLATION OF PROTEINS FROM *S. sanguis* CULTURE SUPERNATANT THAT INHIBIT THE CYTOKINE-INDUCING ACTIVITY OF LPS

7.1 Introduction

Although *S. sanguis* is involved in the inflammatory diseases infective endocarditis and streptococcal septicaemic shock, it is one of the most abundant species of oral commensal, and causes no inflammation in the oral cavity. As an oral commensal, *S. sanguis* must have evolved alongside the human host to eventually arrive at a situation where no significant damage to either party arises. The interesting question to be answered is whether this organism has evolved mechanisms to counteract the host's immune response. Since the inflammatory response is orchestrated by cytokines, a possible strategy for the bacteria would be to interfere with the host cytokine network. The aim of the study described in this chapter was to extract the exported material from *S. sanguis* grown in the absence of blood (to more closely mimic the oral cavity as opposed to the blood-stream) and investigate its ability to interfere with the induction of IL-6 production in PBMC. LPS induces PBMC to secrete a variety of cytokines, including IL-6, and its mechanism of action has been studied extensively. In the experiments described in this chapter, LPS was used as a way of inducing cytokine secretion in PBMC, to allow the screening of *S. sanguis* proteins for the ability to interfere with this cytokine production. The ultimate goal was to isolate the component(s) responsible for any such activity. However, growth of *S. sanguis* on plates was greatly reduced in the absence of blood, making production of large amounts of EM, such as would be required for purification, impractical. Thus the majority of the work described in this chapter involves the isolation of cytokine-modulating proteins from bacterial culture supernatants.

7.2 Materials and methods

7.2.1 Production of EM from *S. sanguis* grown in the absence of blood

Bacteria were grown on agar plates as described in Chapter 3, section 3.2.1, except that the blood supplement was omitted from the medium. EM was extracted from harvested bacteria as described in Chapter 3, section 3.2.2; this material will be termed "blood-free EM" to distinguish it from the EM isolated from *S. sanguis* grown on blood-supplemented agar ("blood-grown EM") (Chapter 3). The protein content was measured using a Bio-Rad DC assay (Chapter 2, section 2.2.1)

7.2.2 Comparison of the protein profiles of blood-grown EM with blood-free EM by SDS-PAGE

Equal amounts of protein (5.5μg) from *S. sanguis* blood-grown EM (Chapter 3) and blood-free EM were separated on 12% SDS-PAGE gels and silver stained to allow comparison of the proteins secreted under these different growth conditions.

7.2.3 Assessment of the effect of *S. sanguis* EM grown in the presence and absence of blood on the cytokine-inducing activity of LPS

A routine PBMC assay was set up as described in Chapter 2, section 2.5 and cells were stimulated with either blood-grown EM or blood-free EM at 0.1 and 1μg/ml protein. LPS was also added to each well to give a final concentration of 10ng/ml. Supernatants were assayed for IL-6 as described in Chapter 2, section 2.6. Various concentrations of blood-free EM were also tested alone to assess the IL-6-inducing activity. In some experiments, PBMC were incubated with various concentrations of blood-free EM, and the viability assessed by trypan blue dye exclusion as described in Chapter 2, section 2.5.3.

7.2.3.1 Assessment of the effect of heat on the cytokine-modulating activity of blood-free *S. sanguis* EM

Blood-free EM (diluted to 1mg/ml in pyrogen-free water) was heated in a boiling water-bath for 30 minutes, and then allowed to cool before adding to cells in a routine PBMC

assay as described in Chapter 2, section 2.5. Blood-free EM that had not been heat treated was also tested. Both the heat-treated and untreated EM were tested at 0.1 and 1μg/ml, and LPS was added to each well at 10ng/ml. The amount of IL-6 in the supernatants from the PBMC assays was assessed by ELISA as described in Chapter 2, section 2.6.

7.2.4 Production of *S. sanguis* culture supernatant protein

Bacterial culture supernatant was prepared and ammonium sulphate precipitated as described in Chapter 6, section 6.2.2.

7.2.5 Assessment of the cytokine-inhibiting activity of protein from *S. sanguis* culture supernatant

The protein preparation from the *S. sanguis* culture supernatant was diluted in PBS, and various concentrations tested in triplicate, both alone, and in the presence of 10ng/ml of *E. coli* LPS. In further experiments the culture supernatant was tested with 50pg/ml *E. coli* LPS, a concentration shown to produce sub-maximal cytokine induction for these preparations of PBMC. In some experiments the supernatant protein was first heat-treated for 30 minutes in a boiling water-bath, before testing in the assay. The amount of IL-6 secreted into the medium by the PBMC was measured by ELISA as described in Chapter 2, section 2.6. The viability of the PBMC was assessed by trypan blue dye exclusion as described in Chapter 2, section 2.5.3.

7.2.6 Isolation of cytokine-inhibiting proteins from *S. sanguis* culture supernatant

Since the *S. sanguis* culture supernatant protein had already been partially fractionated by cation exchange chromatography (Chapter 6, sections 6.2.4 & 6.3.2.5), these fractions were used as a starting point for the isolation of the inhibitory activity. As was described for the isolation of cytokine-inducing proteins (Chapters 5 & 6), a modification of the routine PBMC assay was used as a screen for cytokine-inhibiting activity at each stage of the isolation procedure.

7.2.6.1 General method for the assessment of the ability of bacterial proteins to inhibit LPS-induced IL-6 secretion by PBMC.

The culture supernatant protein appeared to be less potent than the blood-free EM at inhibiting LPS-induced IL-6 secretion from PBMC. A revised method was therefore used for the screening of isolated proteins for this activity, using lower concentrations of LPS. Pooled donor buffy coat residue blood was used as the source of PBMC as this was more readily available than single donor buffy coat blood during this section of the study. PBMC were prepared and the routine PBMC assay followed, as described in Chapter 2, section 2.5. Bacterial proteins or fractions were added to the PBMC at least five minutes prior to the addition of LPS. *E. coli* LPS was used at various concentrations, usually less than 100pg/ml, and in each experiment a dose response of the PBMC preparation to this stimulus was carried out to assess whether the chosen concentration of LPS was sub-maximal for IL-6 production. In experiments involving large numbers of fractions requiring the use of multiple 24-well plates, LPS was included on every plate to facilitate the comparison of samples on different plates by normalisation of the data. Cytokine levels in supernatants were assayed using ELISAs, details of which are given in section 2.6.

7.2.6.2 Normalisation of data

Data was normalised for the generation of graphs, although for statistical analysis the result for each fraction was compared to the LPS tested on the same assay plate (see Chapter 2, section 2.7 for details of the t-tests used).

Normalisation of data was carried out in the following way. The value of the LPS control on the first plate was arbitrarily chosen as the standard against which all other values were normalised. The amount of IL-6 measured for samples on other plates was calculated as a percentage of the LPS control on their own plate, and each value normalised against the value for the LPS control on the first plate. An example calculation is given below:-

IL-6 measured for LPS control on plate 1: 2000pg/ml

IL-6 measured for LPS control on plate 2: 1600pg/ml (taken as 100%)

IL-6 measured for sample X on plate 2: 1200pg/ml (75% of the value for the LPS control on plate 2)

Normalised value for IL-6 for sample X on plate 2: 75% of 2000 = 1500pg/ml

7.2.6.3 Assessment of the cytokine-inhibiting activity of fractions from cation exchange HPLC of *S. sanguis* culture supernatant protein

Fractions obtained from the separation of *S. sanguis* protein using the S20 cation exchange HPLC column (Chapter 6, section 6.2.4 & 6.3.2.5) were tested for their ability to inhibit LPS-induced IL-6 secretion by PBMC. 50µl of each fraction was tested, in triplicate, in the presence of 100pg/ml *E. coli* LPS, as described in section 7.2.6.1. Levels of IL-6 were measured using ELISA as described in Chapter 2, section 2.6. Control wells of 100pg/ml LPS were included on every plate to take into account variations between assay plates.

7.2.6.3.1 Dialysis of the cytokine-inhibiting fraction

To confirm that the inhibiting activity of one of the fractions was not due to the buffer it had eluted in, buffer B (0.02M MOPS pH 6.0 + 1M NaCl) was removed from a small amount of the active fraction by dialysis. 1ml was dialysed at 4°C against 3 x 1 litre of distilled water in a Slide-A-Lyser (Pierce & Warriner, UK) with a 10kDa molecular mass cut-off membrane. After dialysis, 36µl of the active fraction was separated on a 12% gel by SDS-PAGE and silver-stained, and the protein profile compared with a sample of the active fraction that had not been dialysed. Samples were also tested for their ability to inhibit LPS-induced IL-6 production by PBMC to determine whether the activity had been lost during the dialysis procedure. This was carried out as described in section 7.2.6.1, using LPS at 100 pg/ml.

7.2.6.4 Preparation of the cytokine-inhibiting fraction for further purification by cation exchange chromatography

The active fraction (12ml) was concentrated by ultracentrifugation in an Amicon spin-cell using a filtration membrane with a 10kDa molecular mass cut-off, as described in Chapter 2, section 2.8.2. Once concentrated to 3ml, the sample was further concentrated using an Amicon Microcon (10kDa cut-off membrane), before dialysing against 3 x 1 litre of distilled water in a 10kDa cut-off membrane Slide-A-Lyser. All concentration and dialysis steps were carried out at 4°C. A stock solution of 0.2M MOPS pH 6.0 was added to the dialysed sample to bring it to a final buffer concentration of 20mM MOPS.

7.2.6.5 Further fractionation of the cytokine-inhibiting fraction by cation exchange chromatography

A MiniS cation exchange column (details of which are given in Chapter 2, section 2.8.3.1.1) was prepared for use on a SMART System as described in Chapter 2, section 2.8.3.1.3. Buffer A was 20mM MOPS pH 6.0, and buffer B, the elution buffer, was 20mM MOPS pH 6.0 containing 1M NaCl. Initially the flow rate was 400µl/min, with an elution gradient of 0-1M NaCl over 5.8ml. Fractions of 200µl were collected. The fractionation was repeated with a shallower elution gradient of 0-0.3M NaCl over 5.8ml.

7.2.6.6 Preparation of the cytokine-inhibiting fraction for anion exchange chromatography

The fractions containing the majority of the protein eluted from the cation exchange chromatography described in section 7.2.6.5. were pooled (total volume 2.4ml) and dialysed at 4°C against 3 x 1 litre of distilled water in a 10kDa cut-off Slide-A-Lyser. A stock solution of 0.2M Tris pH 8.5 (10 x buffer A) was added to the dialysed sample to bring it to a final buffer concentration of 20mM Tris.

7.2.6.7 Fractionation of the cytokine-inhibiting fraction by anion-exchange chromatography

A MiniQ anion exchange column (for details see Chapter 2, section 2.8.3.1.1) was prepared for use on a SMART System as described in Chapter 2, section 2.8.3.1.3. Buffer A was 20mM Tris pH 8.5, and buffer B, the elution buffer, was 20mM Tris pH 8.5 containing 1M NaCl. The flow rate was 400µl/min, with an elution gradient of 0-0.5M NaCl over 5.8ml. Since the active fraction had bound to the cation exchange column, the active proteins were unlikely to bind strongly to an anion exchange column. For this reason, a high concentration of NaCl was assumed to be unnecessary to elute the proteins that bound. Fractions of 200µl were collected.

7.2.6.8 SDS-PAGE analysis of anion exchange chromatography fractions

Twelve percent SDS-PAGE gels were used to separate 2µl of each fraction, as described in Chapter 2, section 2.3. Proteins were visualised by silver staining.

7.2.6.9 Assessment of the cytokine-inhibiting activity of anion exchange chromatography fractions

Fractions were tested for their ability to inhibit LPS-induced IL-6 secretion by PBMC. Initially 1µl of each fraction was tested, in triplicate, in the presence of 100pg/ml *E. coli* LPS, as described in section 7.2.6.1. Levels of IL-6 were measured by ELISA as described in Chapter 2, section 2.6. As 100pg/ml LPS was shown to be maximal for inducing PBMC to secrete IL-6 in this first experiment and no inhibition was observed, the assay was repeated with 5µl of each fraction in the presence of 30pg/ml *E. coli* LPS (shown to be sub-maximal by means of a dose-response assay). Data was normalised for the generation of the graph as described in section 7.2.7.1, but for statistical analysis fractions were compared to samples of LPS tested on the same assay plate (see Chapter 2, section 2.7 for details of t-Tests).

7.2.6.9.1 SDS-PAGE and electro-blotting of the active fraction

The active fraction was concentrated from 350µl to 23µl in an Amicon Microcon with a 10kDa cut-off membrane. The sample was separated on a 12% gel by SDS-PAGE and blotted onto PVDF membrane (Millipore) as described in Chapter 2, section 2.4.2.

7.3 Results

7.3.1 Protein content of EM

The dry mass of freeze-dried blood-free EM was too low to be accurately weighed and so a percentage yield could not be calculated. The entire EM preparation was dissolved in PBS and the protein content measured using the Bio-Rad DC assay, allowing a percentage yield in terms of protein extracted from a known dry weight of bacteria to be calculated. A percentage yield of 0.14% was arrived at (meaning that each gram of bacteria yielded extracted material containing 0.0014 grams of protein). For comparison, the same calculation was done for two representative batches of blood-grown EM, and yields of 1.19 and 1.06% were found.

7.3.2 Comparison of the protein profiles of blood-grown EM with blood-free EM by SDS-PAGE

Comparison of the EM from bacteria grown on solid media in the presence and absence of 5% horse blood revealed some differences in the protein profiles (figure 7.1).

7.3.3 Effect of blood-grown and blood-free *S. sanguis* EM on the cytokine-inducing activity of LPS

S. sanguis EM extracted from bacteria grown on medium supplemented with blood did not significantly inhibit LPS-induced IL-6 production by PBMC at 0.1 or 1µg/ml protein (equivalent to 1.25 and 12.5µg/ml EM) (Figure 7.2a). In contrast, EM extracted from *S. sanguis* grown in the absence of blood inhibited LPS-induced IL-6 production by 59% ($p<0.01$) when tested at 1µg/ml protein. This was not due to a toxic effect as the viability of PBMC incubated with this EM (measured by trypan blue dye exclusion) remained greater than 95% even at concentrations of up to 5µg/ml. In addition, IL-6

production showed no signs of decreasing until PBMC were incubated with more than 1.25μg/ml EM (figure 7.2b).

7.3.3.1 Effect of heat on the cytokine-inhibiting activity of blood-free EM

Heat treatment (boiling for 30 minutes) blocked the cytokine-inhibiting activity of the blood-free EM (figure 7.3), indicating that the active component(s) were likely to be proteinaceous.

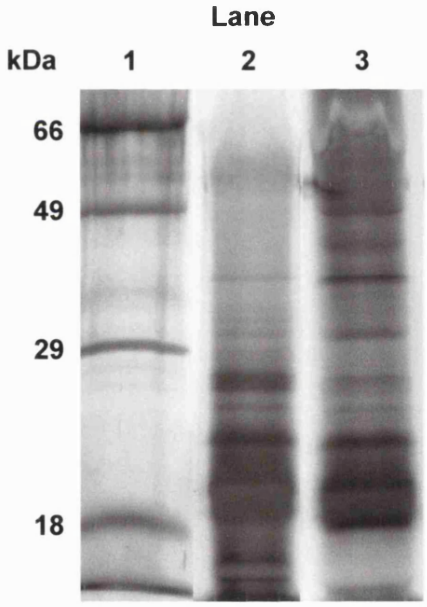

Figure 7.1 Comparison of EM from *S. sanguis* grown as a biofilm in the presence and absence of blood, by SDS-PAGE analysis on a 12% gel. Lane 1 shows the molecular mass markers; lane 2 shows EM from *S. sanguis* grown in the presence of blood (5.5μg protein); lane 3 shows EM from *S. sanguis* grown in the absence of blood (5.5μg protein). The contaminating horse serum proteins detected in the blood-grown EM (Chapter 3) were 27 and 61kDa, but there are differences in proteins other than these.

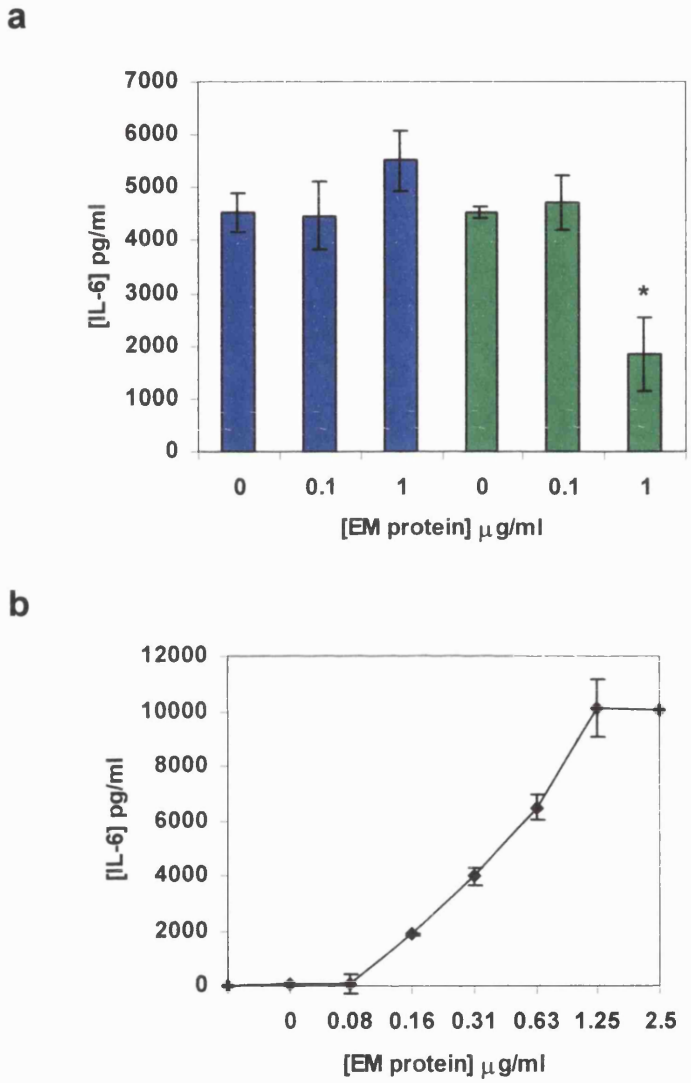

Figure 7.2 Effect of *S. sanguis* blood-grown EM and blood-free EM on the IL-6-inducing activity of LPS. Panel (a) shows IL-6 production by PBMC incubated with LPS at 10ng/ml plus various concentrations of either blood-grown EM (blue bars), or blood-free EM (green bars). *$p<0.01$ when IL-6 production in response to LPS tested in the presence of 1μg/ml EM is compared to LPS alone, using a Student's t-Test. Panel (b) shows IL-6 production by PBMC in response to various concentrations of blood-free EM. Results are expressed as the mean and SD of triplicate cultures.

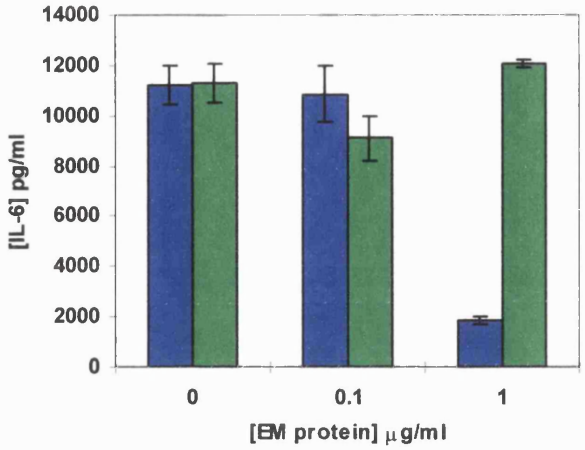

Figure 7.3 Effect of heating on the ability of blood-free EM to inhibit LPS-induced IL-6 production by PBMC. PBMC were stimulated with LPS at 10ng/ml plus various concentrations of un-treated blood-free EM (blue bars) or heat-treated EM (green bars). Blood-free EM was heat-treated by boiling for 30 minutes.

7.3.4 Cytokine-inhibiting activity of protein from *S. sanguis* culture supernatant.

As was described in Chapter 6 (section 6.3.2.2), the culture supernatant protein stimulated PBMC to secrete IL-6 at >0.4μg/ml protein in a dose-dependent fashion, with levels of IL-6 continuing to increase up to 40μg/ml protein, after which the level of IL-6 decreased (figure 6.5a, and repeated here as figure 7.4a to aid the reader). The protein was also tested in the presence of *E. coli* LPS at 10ng/ml, but did not inhibit LPS-induced IL-6 production (results not shown). However, when the protein was tested with a lower concentration of *E. coli* LPS (50pg/ml), sub-stimulatory levels of *S. sanguis* protein (0.4, 0.04 and 0.004μg/ml protein) significantly decreased the level of IL-6 produced by PBMC in response to LPS ($p<0.01$) (figure 7.4b). This was not due to a decrease in cell viability as shown by trypan blue dye exclusion; the percentage viability was not significantly different to that of unstimulated PBMCs, and was consistently greater than 88%. The positive controls of PBMC incubated with 10μg/ml LPS (a concentration shown to be toxic to PBMC plated at this cell density) showed a significantly decreased viability (46%, $p<0.05$). The concentration of LPS that the supernatant protein was able to inhibit (50pg/ml) was shown, by means of a dose-

response experiment, to be a sub-maximal concentration for stimulating PBMC to sectete IL-6 in this assay (figure 7.5).

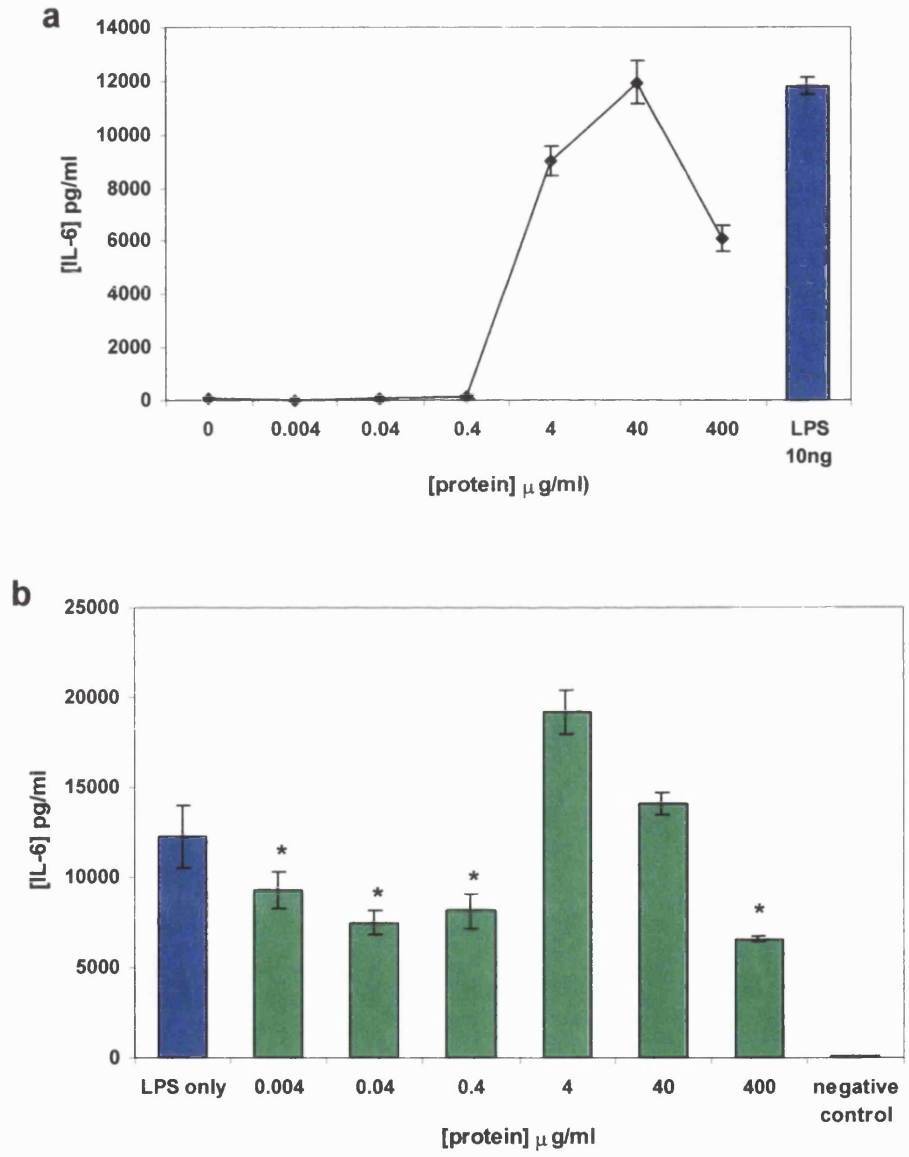

Figure 7.4 Effect of *S. sanguis* culture supernatant protein on cytokine secretion by PBMC. Panel (a) shows IL-6 secretion by PBMC in response to various amounts of *S. sanguis* protein. *E. coli* LPS at 10 ng/ml was tested as a positive control. Panel (b) shows the effect of various amounts of *S. sanguis* protein on LPS-induced IL-6 secretion. All wells were stimulated with LPS at 50 pg/ml except the negative control which contained medium only. Results are expressed as the mean and SD of triplicate cultures. * $p<0.01$ when the amount of IL-6 produced in response to LPS in the presence of *S. sanguis* protein (green bars) is compared to the amount produced in response to LPS alone (blue bar) using a Dunnett's t-Test.

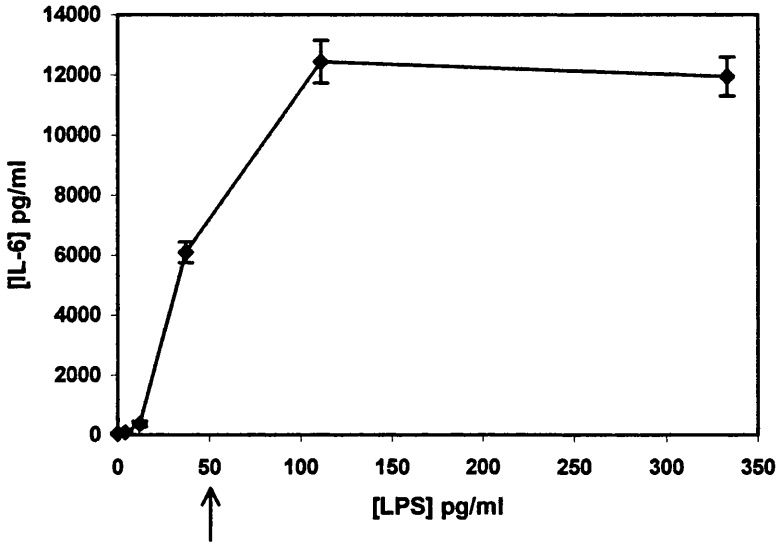

Figure 7.5 IL-6 secretion by PBMC in response to various concentrations of *E. coli* lipopolysaccharide. The arrow indicates the concentration of LPS used in the experiments to assess the ability of *S. sanguis* protein to inhibit LPS-induced IL-6 secretion. The results are expressed as the mean and SD of triplicate cultures.

7.3.4.1 Effect of heat on the cytokine-inhibiting activity of *S. sanguis* culture supernatant protein

As had been demonstrated for the blood-free EM, the cytokine-inhibiting activity of the supernatant protein could be blocked by heating for 30 minutes in a boiling water-bath (figure 7.6).

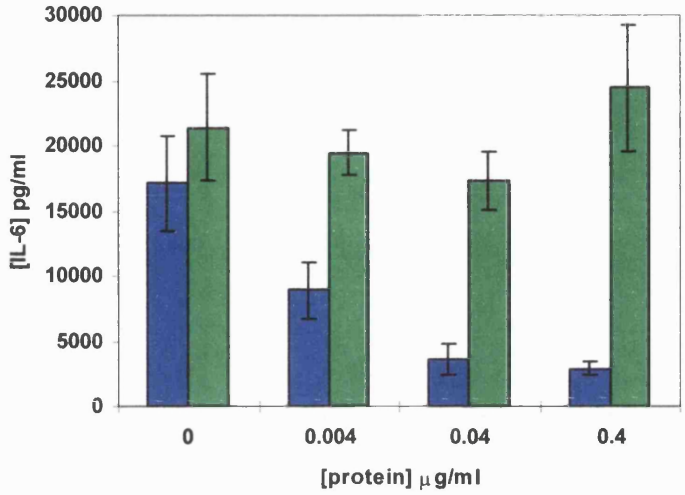

Figure 7.6 Effect of heating on the ability of *S. sanguis* culture supernatant proteins to inhibit LPS-induced IL-6 production in PBMC. PBMC were stimulated with a sub-maximal concentration of LPS (100pg/ml) plus various concentrations of un-treated (blue bars) or heat-treated (green bars) supernatant proteins. The supernatant was heat-treated by boiling for 30 minutes. The results are expressed as the mean and SD of triplicate cultures.

7.3.5 Isolation of cytokine-inhibitory proteins from *S. sanguis* culture supernatant protein

To aid the reader, a flow diagram has been included so that the discussion of the purification schemes described in the remainder of this chapter can be followed more easily (Figure 7.7).

The culture supernatant protein had already been partially fractionated using an S20 cation exchange column in an attempt to isolate cytokine-inducing proteins (Chapter 6). The fractions obtained from this chromatographic separation were thus tested for the presence of cytokine-inhibiting activity.

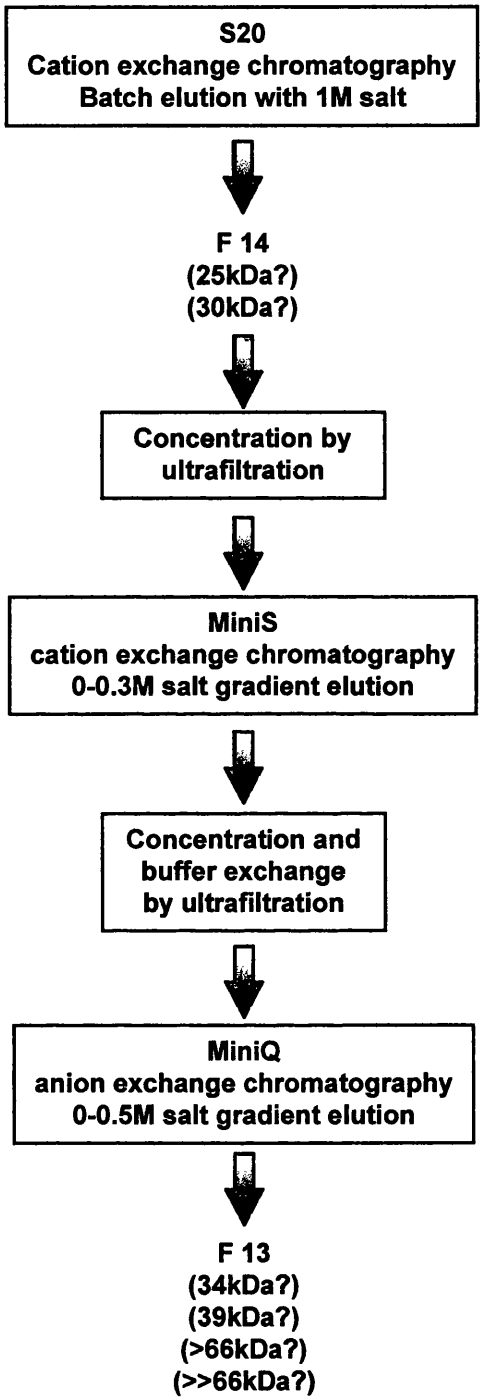

Figure 7.7 Flow diagram of the isolation of cytokine-inhibiting proteins from *S. sanguis* culture supernatant protein. Purification steps are represented by rectangular boxes. Fractions are denoted by F followed by the fraction number. At each stage the molecular mass of the putative IL-6-inducing proteins is given in brackets beneath the appropriate fraction. The question mark indicates the protein(s) thought most likely to be the cytokine-inducing components at that stage in the purification.

7.3.5.1 Cytokine-inhibiting activity of fractions from cation exchange chromatography of protein from *S. sanguis* culture supernatant

Two main groups of fractions significantly inhibited LPS-induced IL-6 production by PBMC: fractions 7 and 8, and fractions 13 and 14 (figure 7.8). The most interesting was fraction 13 (shown as lane 13 in figure 7.9) since it contained a protein of approximately 25kDa in size, which was not present in fractions without the inhibitory activity. This fraction also contained a protein of approximately 30kDa which was only present in small amounts in neighbouring, inactive fractions. However, since buffer B also appeared to down-regulate LPS-induced IL-6 production (figure 7.8, sample 2), it was possible that the inhibitory activity of this fraction was due to the presence of buffer B, rather than either the 25 or 30kDa protein. Before further purification, sample 13 was dialysed to remove buffer B and re-tested to eliminate the possibility of its inhibitory activity being due to buffer B.

7.3.6 Dialysis of the cytokine-inhibiting fraction

Both dialysed and un-dialysed protein significantly inhibited the production of IL-6 from PBMC stimulated with LPS ($p<0.05$ and $p<0.01$ respectively) (figure 7.10a). In addition, both dialysed and un-dialysed protein inhibited LPS-induced IL-6 production to the same degree (i.e. there was no significant difference in the inhibitory activity of the two samples; $p>0.05$). SDS-PAGE analysis of the active fraction confirmed that the protein profile was unchanged by the dialysis procedure: no major proteins were lost (figure 7.10b).

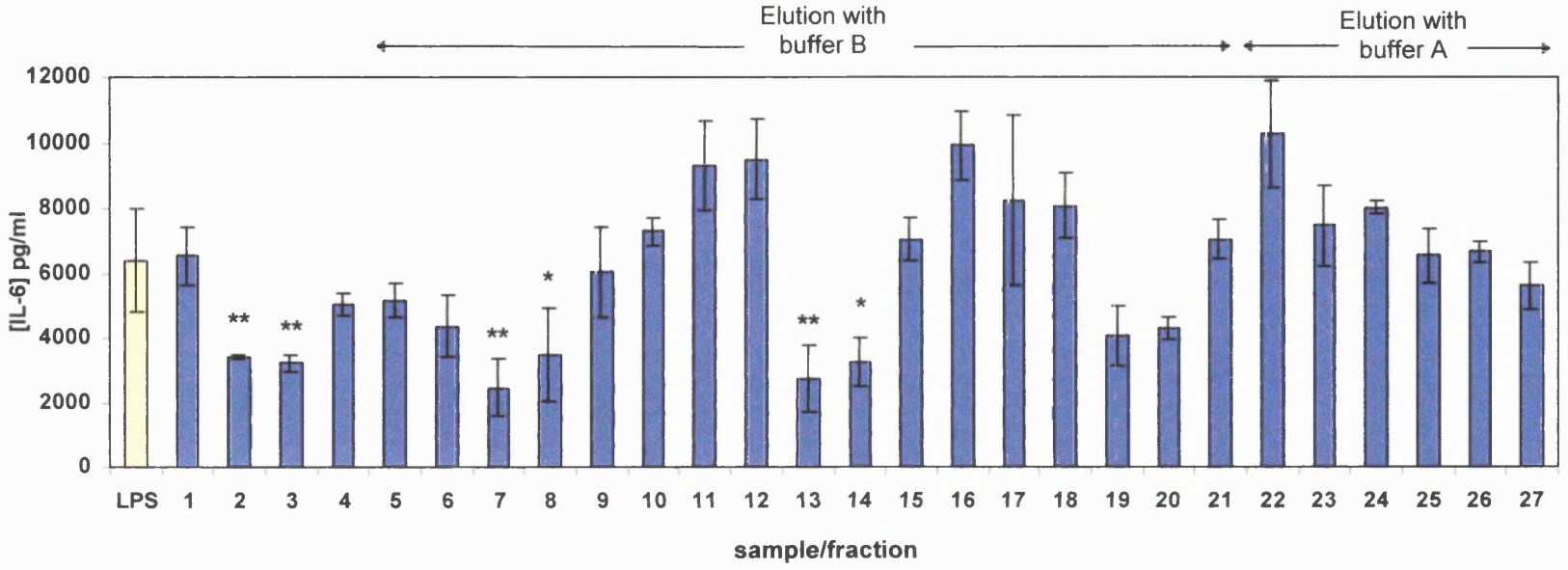

Figure 7.8 Fractionation of *S. sanguis* culture supernatant protein using an S20 cation exchange column: cytokine-inhibiting activity. Data has been normalised for comparison of samples tested on different assay plates. The yellow bar represents the control of LPS only (100pg/ml); sample 1 represents LPS in the presence of buffer A; sample 2 represents LPS plus buffer B; sample 3 represents LPS plus *S. sanguis* protein before fractionation; sample 4 represents LPS plus run-off I (unbound protein from the S20 column); samples 5 to 21 are LPS plus fractions collected during elution with buffer B (each fraction comprising three pooled 4ml fractions); samples 22 to 27 are LPS plus the first 6 fractions collected after the buffer was changed back to buffer A (these are not pooled fractions but individual 4 ml fractions). The remaining fractions had no LPS-inhibiting activity and are not shown here. Sample/fraction numbers 5 to 27 correspond to the lane numbers in figure 7.9. Results are expressed as the mean and SD of triplicate cultures. *$p<0.05$, and **$p<0.01$ when the amount of IL-6 induced by LPS plus a particular fraction is compared to that induced by LPS alone (yellow bar), using Dunnett's t-Test.

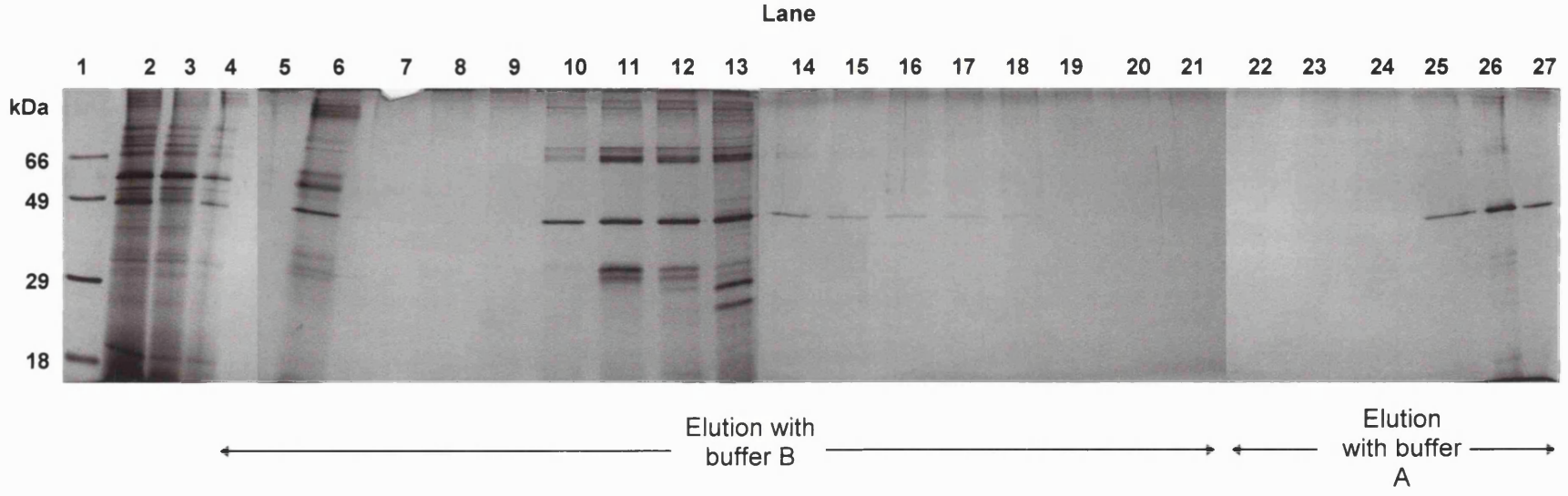

Figure 7.9 Fractionation of proteins from *S. sanguis* culture supernatant using an S20 cation exchange HPLC column. The fractions obtained were run on 12% SDS PAGE gels and silver stained. Lane 1 shows the molecular weight markers; lane 2 shows the protein before separation; lanes 3 and 4 show run-off I and II respectively; lanes 5 to 21 show the fractions collected during elution with buffer B (each fraction comprising three pooled 4ml fractions); lanes 22 to 27 show the first 6 fractions collected after the buffer was returned to buffer A (these are not pooled fractions but individual 4 ml fractions). Lane numbers 5 to 27 correspond to the sample/fraction numbers in figure 7.8. The remaining fractions did not contain any protein bands visible with silver stain and are therefore not shown here.

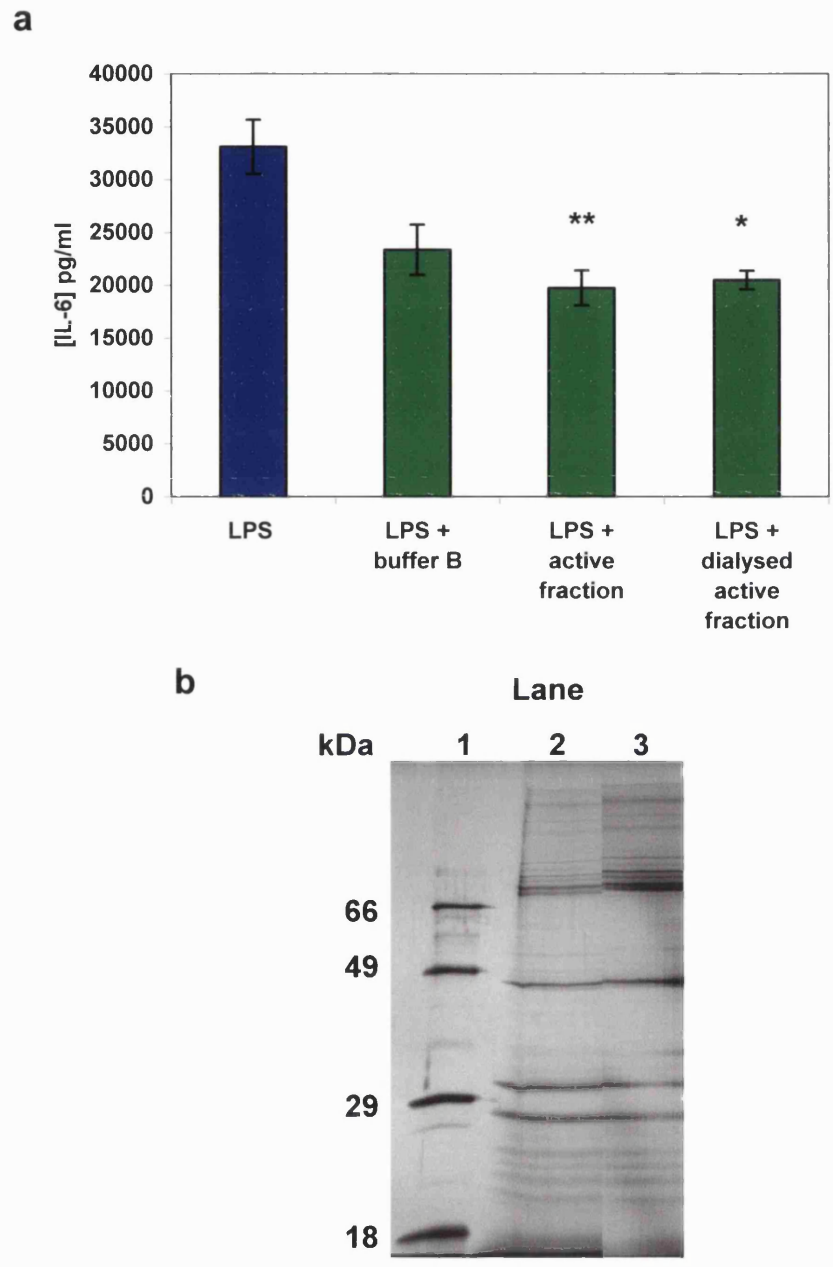

Figure 7.10 Effect of dialysis on the cytokine-inhibiting fraction. Panel (a) shows the inhibition of LPS activity by the active fraction before and after dialysis. *E. coli* LPS was used at 100pg/ml. Results are expressed as the mean and SD of triplicate samples. *$p<0.05$ and **$p<0.01$ when the amount of IL-6 secreted is compared to that secreted in response to LPS alone (blue bar) by a Student's t-Test. Panel (b) shows a picture of the SDS-PAGE analysis of the cytokine-inhibiting fraction before and after dialysis. Lane 1 shows the molecular mass markers; lane 2 shows the active fraction before dialysis; lane three shows the active fraction after dialysis.

7.3.7 Separation of fraction 14 by cation exchange chromatography

Use of a MiniS cation exchange column with an elution gradient of 0-1M NaCl over 5.8ml resulted in all the protein eluting in a single peak in one 200µl fraction. The method was repeated with a shallower salt gradient of 0-0.3M NaCl over the same volume, but the protein still eluted in a single large peak split between two 200µl fractions. The protein eluted at between 0.03 and 0.08M NaCl (data not shown).

7.3.8 Separation of fraction 14 by anion exchange chromatography

Anion exchange chromatography using a MiniQ anion exchange column proved more successful in fractionating the proteins from the LPS-inhibiting fraction. SDS-PAGE analysis of the fractions revealed that most of the proteins had eluted in fractions 10-16 (figure 7.11a). Only one fraction, fraction 13, significantly inhibited IL-6 production by PBMC in response to LPS ($p<0.05$) (figure 7.11b). The proteins originally thought to be responsible for the cytokine-inhibiting activity (the 25 and 30kDa proteins) eluted mainly in fractions 14 and 15, and only to a small extent in fraction 13, and were therefore concluded to be inactive in terms of inhibiting LPS-induced IL-6 production in PBMC. The active fraction (fraction 13) eluted at 0.16M NaCl. This fraction contained four possible candidates for this activity: a 34kDa protein, a 39kDa protein, and two proteins greater than 66kDa, which fall outside the standard curve and so their mass cannot be calculated.

7.3.9 SDS-PAGE and electro-blotting of the LPS-inhibiting fraction

Since there were only four possible proteins responsible for the LPS-inhibiting activity of fraction 13, it was decided to try and obtain N-terminal sequence data for them, rather than proceed to a further purification step and run the risk of losing protein. Unfortunately, on staining the PVDF membrane onto which the protein had been transferred, the protein bands were barely visible and there was insufficient to obtain N-terminal sequence data.

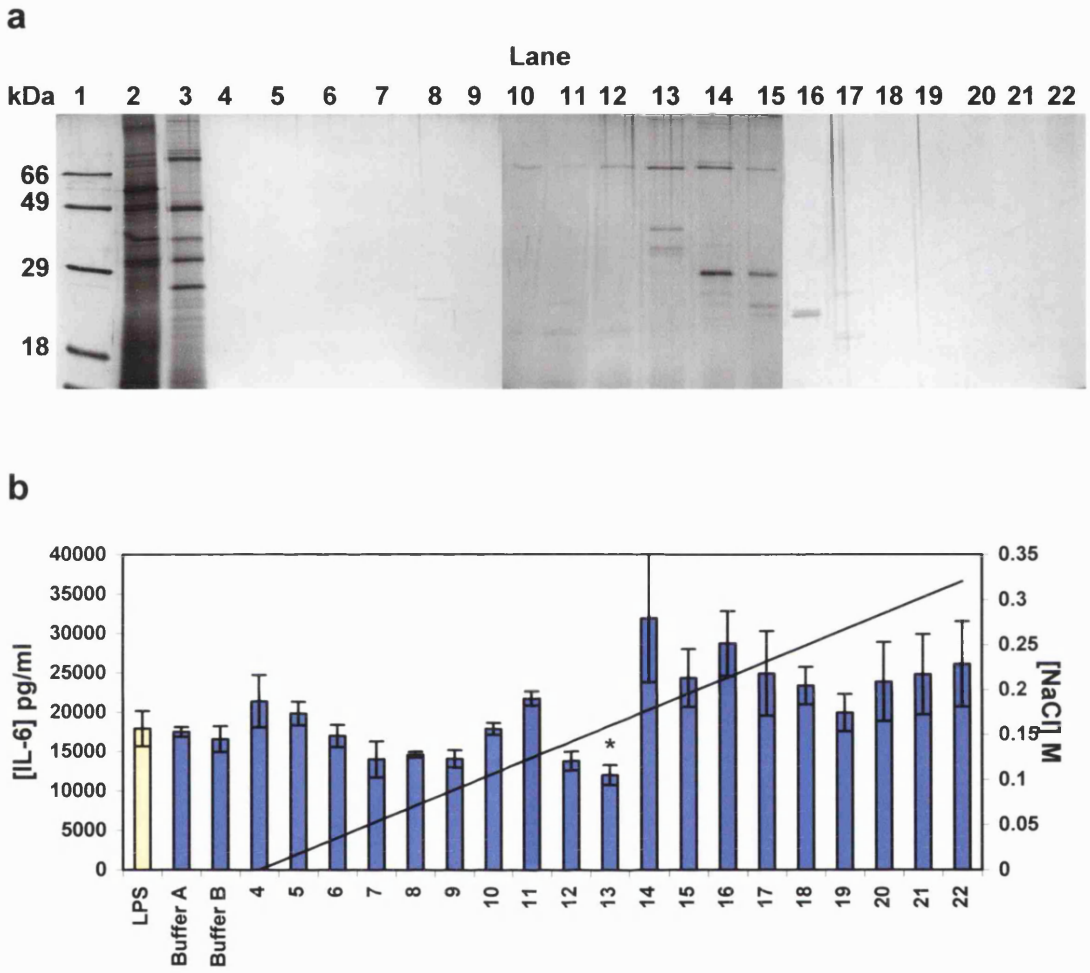

Figure 7.11 Fractionation of active fraction 14 by anion exchange chromatography using a MiniQ column. Panel (a) shows the fractions run on 12% SDS-PAGE gels and silver stained. Lane 1 shows the molecular mass markers; lane 2 shows the original unfractionated ammonium sulphate precipitated protein; lane 3 shows fraction 14 prior to fractionation by anion exchange chromatography; lanes 4 - 22 show fractions eluted from the MiniQ column with a 0-0.32M NaCl gradient. Fractions collected at higher NaCl concentrations contained no protein bands visible with silver stain and are not shown. Panel (b) shows IL-6 produced by PBMC stimulated with various fractions in the presence of a sub-maximal concentration of *E. coli* LPS. The yellow bar represents PBMC stimulated with 100pg/ml LPS only. All other bars represent PBMC stimulated with 100pg/ml LPS in the presence of either buffer A, buffer B, or fractions 4 - 22 (with fraction numbers corresponding to lane numbers in panel (a)). Fractions outside this range were not tested. Results are expressed as the mean and SD of triplicate cultures. *$p<0.01$ when the amount of IL-6 secreted is compared to that obtained for LPS alone (yellow bar) using Dunnett's t-Test.

7.4 Discussion

When EM preparations from blood-grown and blood-free *S. sanguis* were compared in terms of the amount of protein extracted per gram dry weight of bacteria, blood appeared to have a profound effect on the yields. In the absence of blood the percentage yield in terms of protein was reduced from an average of 1.1% to 0.14%, a reduction of 88%. This may simply reflect an overall decrease in the amount of EM produced, rather than a change in the proportional composition of protein. This would seem likely given the fact that the amount of EM produced in the absence of blood was too small to be accurately weighed. The increased mass of the blood-grown EM may be accounted for by contaminating blood proteins, demonstrated to be present in blood-grown EM in the study described in Chapter 3.

When equal amounts (in terms of protein) of the two EM preparations were examined by SDS-PAGE, differences in the protein banding pattern were observed. Some proteins were less abundant in the blood-free EM, while the expression of others appeared to have been upregulated. It is possible that some of the high molecular mass proteins in the blood-grown EM had degraded to smaller protein fragments, thus giving the appearance of the larger proteins being more abundant (or upregulated) in EM produced without blood. However, it seems unlikely that only the blood-grown EM would suffer degradation as both EMs were prepared at the same time, and treated in the same way, although protease production under the different growth conditions was not investigated. These results suggest the regulation of bacterial protein expression by blood components. There is surprisingly little in the literature regarding protein expression in bacteria grown in blood or serum. Ferron and co-workers cultured invasive strains of *Neisseria meningitidis* in laboratory media (iron-restricted and non iron-restricted) and compared the outer membrane protein (OMP) profile with that of bacteria grown in human plasma (Ferron *et al.* 1994). Both growth in plasma and in iron-limited medium induced the expression of new OMPs, including one of the major iron-regulated outer membrane proteins, transferrin-binding protein 2. However, more proteins were upregulated in plasma-grown bacteria suggesting perhaps that conditions in plasma, other than iron limitation, also affected gene expression. In studies by Vallis

and colleagues investigating the growth of *P. aeruginosa* in the presence of serum, secretion of Exoenzyme S, an ADP-ribosyltransferase, was found to be induced when this organism was grown in tissue culture medium containing 10% newborn calf serum (Vallis *et al.* 1999). Most work relating to bacterial gene expression in the bloodstream has focused on culturing bacteria in defined, iron-restricted media, as there is very little free iron in human blood. Iron-regulated protein expression has been reported in several Gram-positive organisms, particularly the staphylococci which are known to produce siderophores to enable iron uptake (Lindsay and Riley, 1994; Cockayne *et al.* 1998; Hill *et al.* 1998; Heinrichs *et al.* 1999). *S. pyogenes* has been demonstrated to release surface glyceraldehyde-3-phosphate-dehydrogenase (also known as the streptococcal plasmin receptor) in response to iron starvation (Eichenbaum *et al.* 1996).

Secreted material extracted from *S. sanguis* grown as a biofilm in the absence of blood (blood-free EM) exhibited cytokine-inhibiting activity: isolated PBMC secreted significantly less IL-6 in response to *E. coli* LPS when tested in the presence of blood-free EM. In contrast, blood-grown EM exhibited no such inhibitory activity. The inhibitory activity of blood-free EM was blocked by heating suggesting that the active components were proteinaceous. Ideally the next step would have been to isolate the active components from the blood-free EM in a similar way to that described in Chapter 5 for the purification of IL-6-inducing proteins. However, in the absence of blood, plate-grown bacterial yields were low and produced only relatively small amounts of EM. As an alternative approach, culture supernatant protein from planktonically-grown *S. sanguis* was investigated for cytokine-inhibiting activity.

Initial results showed that protein from TSBY-grown *S. sanguis* culture supernatant possessed LPS-inhibiting activity, although the supernatant protein was considerably less efficaceous than the blood-free EM. The supernatant protein was only able to inhibit LPS-induced IL-6 production when sub-maximal concentrations of LPS were used. This might have been due to reduced expression of the inhibitory components as a result of the change in growth conditions (i.e. planktonic rather than biofilm-like growth on agar). However, the supernatant protein did possess cytokine-inhibitory

activity, and the inhibition was not due to a toxic effect as cell viability remained greater than 88%. The trypan blue dye exclusion method used to determine cell viability showed a significant decrease in the viability of PBMC incubated with 10μg/ml *E. coli* LPS, included as a positive control to confirm that the trypan blue test could detect cell toxicity. The abolition of inhibitory activity by prior heat treatment confirmed that the activity was due to proteinaceous components, rather than due to contaminating LTA, a molecule that shares usage of CD14 with LPS, and could, in theory, competitively inhibit LPS binding to monocytes.

To try and isolate the LPS-inhibiting component, *S. sanguis* culture supernatant protein was fractionated by cation exchange chromatography using an S20 HPLC column in a batch-type method (i.e. no salt gradient was run). When 50μl of each fraction was tested in the presence of a sub-maximal concentration of LPS in the routine PBMC assay, three groups of fractions showed LPS-inhibiting activity. Fraction 14 contained a 25kDa protein which was not present in fractions without this activity, and so was selected for further purification. However, since the elution buffer used in the cation exchange HPLC fractionation had also exhibited LPS-inhibiting activity, a small amount of fraction 14 was dialysed to remove the elution buffer, and re-tested for activity. The LPS-inhibiting activity was still present even after dialysis, and so the activity was concluded to be due to one or more of the proteins present in the fraction.

Further separation of the proteins in fraction 14 was then attempted. Initially cation exchange chromatography was tried using a MiniS column which gives better resolution than the larger S20 column. Gradients of 0-1M, and then 0-0.3M NaCl were tried but all the protein eluted in a single peak and no further separation was achieved. The protein was then dialysed to remove the elution buffer in preparation for fractionation by anion exchange chromatography using a MiniQ column. Using a relatively shallow NaCl gradient (0-0.5M), the proteins in fraction 14 were separated into about 7 fractions, each containing no more than five or six proteins. Fractions were tested for activity and one fraction, fraction 13, was found to significantly inhibit the production of LPS-induced IL-6 by PBMC. This active fraction eluted at 0.16M NaCl, and contained four protein bands of approximately 34kDa, 39kDa, and two larger proteins,

one of which may be as large as 200kDa in size. Only about 200µl of this active fraction was available, and further purification steps ran the risk of losing even more protein. So, even though a single protein had not been isolated at this stage, it was decided to proceed straight to electro-blotting and attempt to obtain N-terminal sequence data for the four proteins. Unfortunately, there was insufficient protein to sequence even without going through further purification steps, and the attempt used up all remaining active protein. However, partial characterisation had been achieved, and the following facts are known about the active component:-

1. It binds to a cation exchange chromatography matrix - the charged group on both the S20 and MiniS (sulphonate) columns is:-

$R\text{-}CH_2\text{-}SO_3^-$.

The active component elutes from this charged group at approximately 0.03-0.08M NaCl, and so binds relatively weakly to such charged groups at pH6.0.

2. It also binds to anion exchange chromatography matrices at pH6.0 - the charged group on the MiniQ (quaternary amino group) is:-

$R\text{-}CH_2N^+(CH_3)_3$.

The active component elutes from this charged group at approximately 0.16M NaCl.

3. The activity could be due to one of four proteins:-

34kDa

39kDa

A protein greater than 66kDa

A very large protein, possibly as large as 200kDa

Unfortunately there was not enough time to produce more bacterial culture supernatant and repeat this purification. If more time was available and a larger amount of material produced, the four proteins could be further separated by gel filtration chromatography which fractionates proteins on the basis of their size. Purification of amounts of active protein large enough for use in further studies (for example on its mechanism of action)

would be impractical, as the protein is only present in small amounts in the culture supernatant. Ultimately, the aim would be to isolate enough of the individual protein (or proteins) responsible for the LPS-inhibiting activity to obtain an N-terminal sequence, or identify using MALDI-TOF mass spectrometry, and then move on to clone and express the protein so enough could be produced for further studies.

CHAPTER 8

GENERAL DISCUSSION

8.1 Introduction

The treatment of infective endocarditis remains a problem for the medical profession, and with the continued emergence of antibiotic resistance amongst causative bacteria, alternative therapies need to be investigated. There is still a great deal to be learned about the pathogenic mechanisms involved in infective endocarditis. Most research to date has focused on the interactions between bacteria and platelets and the development of the characteristic thrombotic vegetation. The subsequent inflammation and tissue damage that occurs has not been studied to any great extent and investigation into this process may provide vital clues for the development of alternative treatments.

As "orchestrators of the immune response", cytokines are now generally accepted to play a crucial role in all infectious disease processes. In addition to co-ordinating the host's defence against invading microorganisms, several cytokines have been implicated in host tissue pathology. For example, over-production of the so-called "pro-inflammatory" cytokines, TNF, IL-1, IL-6 and IL-8, has been implicated in the pathology of rheumatoid arthritis and septic shock. Septic shock, whether of Gram-negative or Gram-positive origin, is driven by the inflammatory cytokine response to bacterial components (LPS in the case of Gram-negative septic shock, and possibly LTA and peptidoglycan in the case of Gram-positive septic shock). Attempts to control septic shock by killing the microbes with antibiotics have been suggested to aggravate the disease by releasing further bacterial components into the system (Periti and Mazzei, 1999).

The hypothesis proposed in this thesis is that the bacteria involved in infective endocarditis contribute to the inflammatory tissue pathology by eliciting a damaging inflammatory cytokine response from host cells. The driving-force behind cytokine production is proposed to be proteins secreted by the causative bacteria.

8.1.1 The importance of being exported

Many groups have identified components of Gram-positive bacteria that elicit an inflammatory cytokine response from human cells. Much of the literature relates to structural components such as LTA or peptidoglycan which are released largely as a result of cell death, and also to a limited extent from live bacteria (Wicken *et al.* 1982). However, the secretion of molecules from bacteria can be finely regulated and, as such, represents a more controlled mechanism for communication between bacterial and host cells. There are likely to be many factors involved in the generation and perpetuation of inflammation in infective endocarditis, not least of which will be the role of platelets. But this does not preclude a role for secreted bacterial proteins which, by their very nature, are capable of affecting many more cell types than the relatively immobile bacteria themselves.

In the work described in this thesis, secreted, or "exported material" has been extracted from *S. sanguis* (one of the most common causal agents of infective endocarditis), and shown to elicit the production of inflammatory cytokines from human PBMC. Since protein expression in bacteria is modified according to the mechanism of growth, *S. sanguis* was initially cultured as a biofilm on agar plates, as opposed to being grown planktonically, to mimic more closely the *in vivo* situation. Protein secreted into the culture supernatant of planktonically-grown bacteria (secreted molecules in the more traditional sense), was also tested for cytokine-modulating activity. Finally, the affect of growth in the presence and absence of blood on the cytokine-modulating activity was investigated.

8.2 Summary of the findings of this thesis

8.2.1 *S. sanguis* Exported Material (EM)

Transmission electron microscopy of *S. sanguis,* grown on agar supplemented with blood, revealed a large amount of extracellular material (EM) that was removed by simply washing the bacteria in saline. This material accounted for 5% of the bacterial dry weight, and was approximately 20% protein, 4% carbohydrate, and less than 10%

lipid. The extract contained at least 45 and 50 proteins, as demonstrated by two-dimensional SDS-PAGE, ranging from 14 to greater than 100kDa. Endotoxin contamination (with potential for inducing cytokine release from PBMC) was estimated to be only 0.00002%. This meant that more than 50μg/ml EM would have to be added to the routine PBMC assay before this contamination would be expected to induce a cytokine response from the PBMC (10pg/ml LPS is borderline for stimulation in this assay (results from the Eastman Dental Institute)). The rest of the material was thought likely to be molecules such as lipoteichoic acid and peptidoglygan, which would probably have been underestimated in the assays used.

8.2.1.1 Cytokine-inducing activity

The EM was found to stimulate, in a dose-dependent manner, human PBMC to release IL-1β, IL-6, IL-8 and TNFα. Induction of IL-1β, IL-6 and TNFα secretion required approximately 300ng/ml EM, while IL-8 was induced by as little as 30ng/ml. IL-8 was produced in very large amounts, reaching 500ng/ml in the culture supernatant, while IL-1β, for example, only reached a maximum of 6.5ng/ml. However, the biological relevance of this difference may be less significant when we consider that IL-8 is active in the nanomolar range, whereas 1000 to 10,000-fold less IL-1β is required to exert its biological effects. For comparison, a commercial preparation of *S. sanguis* lipoteichoic acid was tested for activity in the same assay, and was found to be more potent than the EM, inducing a cytokine response from PBMC at a concentration of 10ng/ml, (although this was comparing a crude mixture with an isolated component, and so may not be a true reflection of the activity of individual components of the EM).

It may be useful at this point to compare the potency of the EM with that of other bacterial components in terms of cytokine-induction. There is now a bewildering number of cytokine-inducing molecules, or "Modulins" originating from bacteria (Henderson *et al.* 1996a; Henderson and Wilson, 1996b). To simplify things a little, only reports pertaining to components of streptococci, plus a few examples from the staphylococci, have been used for comparison, and are shown in table 8.1. Although obviously the experimental conditions vary from study to study, the activity of bacterial

components as measured *in vitro* provides some indication of their relative potencies. However, unless the cytokine-inducing molecule reaches its target cell, none of this will be relevant *in vivo*, and it is difficult to estimate the extent to which this will occur for each different component.

Table 8.1. Comparison of the cytokine-inducing potency of *S. sanguis* EM (from this study) with that of various components of streptococci and staphylococci.

Species	Component	Cytokines induced	Minimum active concentration (ng/ml)	Reference
S. pneumoniae	pneumolysin	IL-1β, TNFα	0.003	(Houldsworth *et al.* 1994)
S. pyogenes & *Staph. aureus*	LTA	IL-8	10	(Standiford *et al.* 1994)
Staph. aureus	SEB & TSST-1	IL-12	100	(Leung *et al.* 1995)
S. mitis	culture supernatant protein	IL-1, IL-6, TNFα, IFNγ	200	(Takada *et al.* 1993)
S. pyogenes	SpeA	IL-1, IL-6, IL-8, TNF	200	(Muller-Alouf *et al.* 1994)
S. sanguis	**EM**	**IL-1β, IL-6, IL-8, TNFα**	**30-300**	**Results presented in this thesis**
Staph. aureus	protein A	IL-1α, IL-4, IL-6, TNFα, IFNγ	1000	(Tufano *et al.* 1991)
S. sanguis & *S. pneumoniae*	LTA	TNFα	1000	(Keller *et al.* 1992) *
Staph. aureus	soluble PG	IL-1, IL-6	3000	(Weidemann *et al.* 1994)
S. mutans (serotype f)	serotype f polysaccharide (poly f)	IL-1β, TNFα	10,000	(Benabdelmoumene *et al.* 1991)
S. mutans (serotype f)	SR protein (member of the antigen I/II family)	IL-1β, IL-6, TNFα	10,000	(Soell *et al.* 1994)

All the studies chosen for comparison report data from experiments with either human PBMC or isolated blood-monocytes, except *, in which bone-marrow-derived mononuclear phagocytes were used. Abbreviations: LTA, lipoteichoic acid; SEB, staphylococcal enterotoxin B; TSST-1, toxic shock syndrome toxin 1; SpeA, *S. pyogenes* erythrogenic toxin A; EM, exported material; PG, peptidoglycan.

The use of a commercial cDNA array kit to investigate the range of cytokine and cytokine receptor genes affected by *S. sanguis* EM, revealed upregulation of various mRNAs. Most were for proteins involved in the generation of inflammation such as IL-1β, IL-8, IL-17 and G-CSF. Notable absences in relation to pro-inflammatory responses included IL-6, TNFα and chemokines such as MIP-1α and MCP-1. Possibly of greater significance was the lack of upregulation of mRNA for any of the cytokines purported to inhibit inflammation, namely IL-1ra, IL-4, IL-10, IL-13 and TGFβ, suggesting that the EM selectively modulates the inflammatory network in a damaging fashion. However, since the data presented represents a single time-point, these genes may turn out to be expressed at other time-points after stimulation.

8.2.1.2 Kinetics of cytokine induction

The results of time course experiments with *S. sanguis* EM suggested that IL-8 and TNFα secretion was induced first, before IL-1β and IL-6. IL-1β, IL-6 and TNFα release began to level off after 20 hours, possibly due to inhibition by some other regulatory molecule (perhaps IL-10 or IL-4), while the level of IL-8 continued to increase. The continued production of a chemokine such as IL-8 *in vivo* might represent a continued attempt by the host to eliminate the source of the infection by recruiting more and more leukocytes. Once the bacteria have been eliminated, the bacterial signals activating the immune response will be removed, production of molecules such as IL-8 will cease, and leukocytes can leave the area or apoptose. In the *in vitro* studies that have been described in this thesis, the fate of the cytokine-inducing molecules of EM is not known (i.e. whether or not they are removed from the system by leukocyte-uptake for example). If they are not removed from the experimental system, then this might explain the continued increase in IL-8 release from PBMCs. If the unregulated production of IL-8 in response to *S. sanguis* EM is not solely an *in vitro* phenomenon, it might have relevance to the chronic inflammation observed in heart valve tissue on which bacterial vegetations have formed. In a situation such as infective endocarditis in which the infecting organisms are difficult to eliminate, the continued influx of leukocytes in response to bacterial-induced IL-8 could be very damaging to tissues.

8.2.1.3 Mechanism of action of EM

The activity of the *S. sanguis* EM was demonstrated to be due to a proteinaceous component by the observation that the activity was substantially and significantly decreased by heating and trypsin digestion. Contamination by the potent cytokine-inducing molecule, LPS, was less than 0.00002%, as measured by the LAL assay. In addition, the LPS-blocking antibiotic, polymyxin B, did not block the cytokine-inducing activity of the EM, even at a concentration of antibiotic demonstrated to block levels of LPS greater than that estimated to be present in the EM at the concentrations tested.

One of the more interesting observations in this study was the apparent requirement for the CD14 receptor in the activation of PBMC by EM. Aside from LPS, a number of other bacterial molecules have been demonstrated to exert their effects via CD14, including peptidoglycan (PG) (Weidemann *et al.* 1994; Gupta *et al.* 1996; Weidemann *et al.* 1997; Dziarski *et al.* 1998), LTA (Cleveland *et al.* 1996; Hattor *et al.* 1997), lipoarabinomannan (Means *et al.* 1999), and rhamnose glucose polymers found on the cell walls of oral streptococci (Soell *et al.* 1995). CD14 has therefore been suggested to be a pattern-recognition receptor, allowing cells to recognise components from both Gram-positive and Gram-negative organisms (Pugin *et al.* 1994). Although LTA and PG share the usage of CD14 with LPS, the LPS receptor complex comprises CD14, the Toll-like receptor 4 (TLR4) and MD2 (Shimazu *et al.* 1999), whereas LTA and PG have been shown to require TLR2 in complex with CD14 to mediate their effects (Schwandner *et al.* 1999). It would be interesting to investigate which (if any) of the TLR receptors are required for the activation of PBMC by *S. sanguis* EM, and thus single out a molecular target for therapy.

8.2.1.4 Isolation of the active components of *S. sanguis* EM

Various purification techniques were used to try and isolate the cytokine-inducing proteins in the EM. After each stage in the purification, fractions were screened for IL-6-inducing activity using human PBMC isolated from single-donor buffy-coat blood. The progress of the purification was followed by separation of the proteins on SDS-PAGE gels. Purification using anion exchange chromatography led to the isolation of a

fraction containing several proteins. Only one protein was unique to this active fraction, and a database search on the N-terminal sequence revealed that it was homologous to the mangenese-dependent superoxide dismutases (SOD) of *S. mutans* and *S. pneumoniae*. Whether or not the SOD was actually responsible for the IL-6-inducing activity of the isolated fraction has not yet been established; the IL-6-inducing activity of a commercial preparation of manganese-dependent SOD from *B. stearothermophilus*, appeared to be due to contaminating LPS. However, there is no evidence that the commercially available protein was biologically active and so these results do not rule out SOD as a cytokine-inducing molecule. With more starting material (and more time), the proteins in the active fraction could be further separated and the active protein identified. Alternatively, the *S. sanguis* SOD gene could be cloned and expressed to determine the protein's IL-6-inducing activity.

A combination of anion exchange and gel filtration chromatography led to the isolation of a second active fraction, this time with the protein purified to homogeneity. Unfortunately this 190kDa protein was not present in large enough amounts to obtain N-terminal sequence data by EDMAN degradation. The possibility that the protein might be related to two of the known adhesins of the oral streptococci was ruled out by the failure of this protein to react with antibodies to the antigen I/II proteins (a family of immunologically and structurally related cell surface adhesins widely expressed among the oral streptococci (Jenkinson and Demuth, 1997a), or antibodies to CshA/CshB of *S. gordonii*. Again, if more time were available, a larger amount of EM could be extracted and purified to yield enough protein for N-terminal sequence analysis. An alternative method of identifying isolated proteins is the use of trypsin digestion and MALDI-TOF mass spectrometry. Unfortunately I did not have access to this technique during my PhD.

8.2.2 *S. sanguis* culture supernatant proteins

The main proteins responsible for the cytokine-inducing activity of the EM appeared to comprise only a very small part of the crude material, leading to problems in obtaining

enough to obtain N-terminal sequence data. It was therefore decided to grow the bacteria as a planktonic culture which would be easier to scale up.

For the production of culture supernatant for purification, *S. sanguis* was grown to stationary phase in TSBY (a medium determined to be suitable on the basis of a pilot study). The resulting bacterial supernatant induced PBMC to release IL-6 at between 400 and 4000ng/ml protein and, as such, was less potent than the EM (which was active at 300ng/ml EM - equivalent to approximately 100ng/ml protein), but since it was relatively easy to produce more culture supernatant, this growth medium was used.

8.2.2.1 Purification of the cytokine-inducing components from *S. sanguis* culture supernatant

A combination of cation exchange chromatography (using a batch elution method) and gel filtration chromatography led to the isolation of an active fraction containing several high molecular mass proteins. Even at this stage there proved to be too little of any of the putative IL-6-inducing proteins to obtain N-terminal sequence data. The majority of the proteins had not bound to the cation exchange column during the first purification step, and these retained substantial IL-6-inducing activity. Attempts were made to purify the active proteins using anion exchange chromatography, but no real leads were found, and so this was abandoned.

The purification of active proteins from either EM or culture supernatant proved difficult due to the small amounts of active components present. One alternative approach to the identification of cytokine-inducing proteins would involve screening sera from patients with infective endocarditis caused by *S. sanguis*. Any patient sera that was able to block EM-induced cytokine production could then be used to screen an *S. sanguis* gene expression library. This method, however, relies on the cytokine-inducing proteins of *S. sanguis* being immunogenic.

8.2.3 Cytokine-inhibitory activity of *S. sanguis* exported material

One of the hypotheses in this thesis was that blood components might be important environmental factors in regulating bacterial expression of cytokine-inducing molecules (modulins), and that this might be relevant to an oral commensal becoming pathogenic in the context of infective endocarditis. It was therefore of particular interest in this study to find that exported material from *S. sanguis* grown on agar without blood, inhibited the IL-6-inducing activity of *E. coli* LPS in human PBMC.

Exported material from *S. sanguis* grown on agar supplemented with blood (blood-grown EM) was compared with an identically prepared extract from the same organism grown in the absence of blood (blood-free EM). Blood affected the yield of protein - the yield was reduced by almost 90% in the absence of blood - and altered the protein banding patterns on SDS-PAGE gels. This suggested that the expression of several extracellular proteins of *S. sanguis* was regulated by blood components.

The ability to inhibit LPS-induced IL-6 production in PBMC was observed only in blood-free EM: the activity of LPS (10ng/ml) was decreased by almost 60% while cell viability remained above 95%. No such activity was observed when the same concentrations of blood-grown EM were tested. In the absence of blood, bacterial yields on solid medium were greatly reduced, so planktonic culture was turned to again, as a means of producing larger amounts of bacterial protein. Protein from the supernatants of overnight cultures of *S. sanguis* inhibited LPS-induced cytokine secretion, albeit at a reduced level to that seen with EM (IL-6 was reduced by 40% compared to 60% with EM). The inhibitory activity was blocked by heating, ruling out the possibility that contaminating LTA or PG (both of which bind to CD14) was preventing LPS from binding to CD14. Attempts to isolate the inhibitory protein using cation exchange chromatography, followed by anion exchange chromatography, led to the isolation of a cytokine-inhibiting fraction which reduced LPS-induced IL-6 release by approximately 33%. At this stage protein had already been lost, and so the candidate proteins were electro-blotted in the hope of obtaining sequence data. Once again there

was insufficient protein to sequence, and no more time was available to repeat the purification with more starting material.

The cytokine response of host cells to microbes is an innate response, and represents an early warning signal of invasion, allowing the host to respond quickly, without requiring the development of acquired immunity. It is unsurprising, therefore, that several bacterial species have evolved mechanisms to inhibit cytokine responses. For example, a 45-50kDa protein from *Brucella suis* has been demonstrated to inhibit TNFα secretion from *E. coli*-infected macrophages (Caron *et al.* 1996). TNFα release from LPS-stimulated murine macrophages is inhibited by YopP from *Yersinia entercolitica* (Boland and Cornelis, 1998). *Por. gingivalis*, an organism implicated in the periodontal diseases, has been demonstrated to hydrolyse IL-1, IL-6 and IL-lra, both when grown planktonically and as a biofilm (Fletcher *et al.* 1997; Fletcher *et al.* 1998).

8.2.3.1 Mechanisms of action of the cytokine-inhibitory activity

It is important to realise that the detection of a decreased amount of IL-6 by ELISA, does not necessarily translate to a decreased bioactivity either *in vitro* or *in vivo*. IL-6 exerts its effects via a receptor complex on the target cell membrane, comprised of the IL-6 receptor (IL-6R) and gp130, the critical component for signal transduction (Taga and Kishimoto, 1997). IL-6 binds to IL-6R, and then the IL-6/IL-6R complex associates with gp130 which is induced to homodimerise. It is thought that signal transduction occurs through the gp130 homodimer, by the activation of a cytoplasmic protein tyrosine kinase of the Janus kinase (JAK) family, and tyrosine phosphorylation and activation of the cytoplasmic transcription factors known as signal transducers and activators of transcription (STATs). Although gp130 is ubiquitously expressed, IL-6R exhibits more limited expression. However, the extracellular soluble form of IL-6R (sIL-6R) can bind IL-6, induce dimerisation of gp130, and function through these homodimers, bypassing the need for membrane-bound IL-6R. The IL-6/sIL-6R complex is therefore able to confer IL-6-responsiveness on cells which express gp130, but not IL-6R. This may be relevant to the apparent inhibitory activity of components from *S. sanguis* culture supernatant protein. If the protein induces upregulation of sIL-

6R from some cell type present in the assay, then any induced IL-6 might not be detected by ELISA as a result of being complexed with sIL-6R (Sehgal, 1996). Streptolysin O from *S. pyogenes* and the *E. coli* haemolysin, (both pore-forming toxins) have been shown to induce the release of sIL-6R from human monocytes and macrophages, and the liberated receptors were demonstrated to be biologically active (i.e. they conferred IL-6-responsiveness on cells that did not express IL-6R) (Walev *et al.* 1996). Complexing of IL-6 with sIL-6R would be observed as a decrease in IL-6 by ELISA measurement, but in terms of biological activity, complexes of IL-6/IL-6R could bind to gp130-expressing cells and exert an effect. For this reason, in addition to the direct measurement of IL-6 by ELISA, it might also have been useful to determine the biological activity of the supernatants from the PBMC assays by bioassay.

Thinking along similar lines to the IL-6/sIL-6R hypothesis, it may be that LPS successfully induces an IL-6 response, but that the active component in the culture supernatant protein of *S. sanguis* is able to sequester IL-6 (for example, it may be an analogue of sIL-6R).

Assuming for the time being that there is an actual decrease in the amount of IL-6 secreted by PBMC in response to LPS, there could be several mechanisms by which the cytokine-inhibitory protein functions. The active component may prevent LPS binding to the LPS receptor complex (CD14 and the Toll-like receptor 4 (TLR4)), either by binding to the LPS molecule, or to part of the receptor complex. Alternatively, inhibition might occur at the level of IL-6 production/release, and the potential points of intervention are listed in Table 8.2.

Table 8.2. Points of intervention potentially exploited by the LPS-inhibiting component of *S. sanguis*.

1. Inhibition of intracellular signalling pathways
2. Inhibition of cytokine gene transcription
3. Interference with mRNA processing or stability
4. Inhibition of cytokine mRNA translation
5. Interference with post-translational modification or secretion

Another possibility is that the anti-inflammatory cytokines IL-4 or IL-10 are induced by supernatant protein from blood-free cultures of *S. sanguis* . IL-4 and IL-10 block the LPS-induced production of various cytokines, including IL-1β, IL-6, IL-8, TNFα and IL-12 (Fiorentino *et al.* 1991; Chomarat and Banchereau, 1997).

LPS has been demonstrated to induce IL-6 indirectly, via the induction of IL-1. Experiments carried out by Granowitz and colleagues demonstrated that the IL-1 receptor antagonist (IL-1ra) reduced the amount of endotoxin-induced IL-6 produced by human monocytes *in vitro* (Granowitz *et al.* 1992a; Granowitz *et al.* 1992b). These results were in agreement with those from the *in vivo* studies of Henricson and colleagues (Henricson *et al.* 1991). Interference with IL-1 induction thus represents another mechanism by which the inhibitory component of *S. sanguis* might exert its effect.

The culture supernatant protein of *S. sanguis* itself induces IL-6 from PBMC, but whether this occurs via IL-1 or not, has not yet been established. If we suppose for a moment that induction of IL-6 by this protein does not have a requirement for prior production of IL-1, then an inhibitor that blocks IL-1 induction, would inhibit LPS-induced IL-6, but not supernatant protein-induced IL-6. Taken together, these suppositions could explain why low concentrations of supernatant protein inhibit LPS-induced IL-6, while at higher concentrations the stimulatory components of the same protein induce IL-6 production without being affected by the inhibitory components.

8.2.3.2 Regulation of expression of the secreted cytokine-inhibitory activity

Preliminary results suggested that the cytokine-inhibitory activity was not expressed when *S. sanguis* was grown in the presence of blood. Whether this might reflect differential gene expression *in vivo* or not is unclear. *S. sanguis*, in the oral setting, does not induce an inflammatory response, and yet in the context of infective endocarditis it causes a disease with inflammatory pathology. It is likely that the change in environment from oral cavity to blood-stream will alter the expression of many bacterial genes (potentially including those controlling the cytokine-inhibiting activity), and this may have implications in terms of how the host responds to the invading organisms (i.e. to what extent inflammation results).

The most obvious changes that *S. sanguis* would experience in the initial events leading to infective endocarditis or septicaemia, are those associated with relocation from the oral cavity to the bloodstream. Aside from exposure to blood components, the bacteria will also be exposed to temperature and pH changes, and any or all of these factors might trigger the expression of different sets of genes. There are many examples of bacterial protein expression being regulated by the surrounding environment. One example of particular relevance to infective endocarditis is the work of Knox and colleagues (Knox *et al.* 1985), who investigated the effect of pH change on extracellular protein expression by a strain of *S. sanguis* grown in a chemostat. A change from pH 5.5 to 7.5, used to simulate the transition from the oral cavity to the bloodstream, resulted in a doubling of total extracellular protein. In addition, there were pronounced differences in the patterns of proteins produced at the two pHs: proteins present in very small amounts (or absent) at one growth pH were major proteins at the other growth pH. In another example of environment-induced phenotype alteration, the expression of the platelet aggregation associated protein (PAAP) of *S. sanguis* was reported to be upregulated by growth in the presence of collagen (Erickson and Herzberg, 1995), or by heat shock (Liu *et al.* 1995). If with more time, the protein responsible for the LPS-inhibition could be identified, it would then be of interest to investigate the environmental factors influencing expression. If, as is indicated by the

preliminary results, the inhibitory activity is regulated by some component of blood, this might help to explain the absence of inflammatory pathology in the oral setting.

8.2.3.3 A role for cytokine-inhibiting activity in bacterial survival?

Assuming for a moment that this inhibitory activity is expressed during growth of *S. sanguis* in the oral cavity, an ability to inhibit the inflammatory response induced by other species of bacteria, could be advantageous to an organism that itself does not induce an inflammatory response. The oral cavity is heavily colonised by Gram-negative organisms, many of which are associated with the periodontal diseases, a collection of diseases driven by inflammatory processes and in which a pathological role for cytokines has been postulated (Wilson *et al.* 1996). The actions of molecules such as LPS, released from these organisms, will trigger an inflammatory response in the host which represents a potential threat to the survival of other organisms such as *S. sanguis* that are growing in close proximity. If *S. sanguis* can modulate the host cells' responses in the immediate environment, it may be better able to survive in an otherwise fairly hostile situation.

S. sanguis itself possesses various potentially inflammatory molecules, including peptidoglycan and LTA, and yet does not induce an inflammatory response in the oral environment. It is possible that, in addition to its effect on LPS activity, the cytokine-inhibiting activity also serves to decrease the inflammatory-inducing activity of molecules from *S. sanguis*. The ability of this material to inhibit the cytokine-inducing activity of other bacterial components (such as LTA) has not yet been investigated, so it is unclear whether such activity has the potential to reduce an inflammatory response induced by *S. sanguis*. The data presented in this thesis suggest that inhibition of the cytokine-inducing activity of molecules from *S. sanguis* does not occur, as both the blood-free EM and the culture supernatant induced significant amounts of IL-6. However, the conditions under which the bacteria were grown in this study are considerably different to those that would be encountered in the oral cavity. The take-home message should be that if an inhibitory molecule exists, then it has the capacity to

be upregulated or down-regulated, depending on the environment, and may, therefore, play a significant biological role *in vivo*.

8.3 A bacterial balancing act

In Chapters 6 and 7 the exported culture supernatant proteins from planktonically-grown *S. sanguis* were investigated for their cytokine-modulating properties. It may at first appear to be somewhat contradictory that bacteria grown under the same conditions produced both elements which stimulated immune cells to produce inflammatory cytokines (Chapter 6), and elements which inhibit an inflammatory response to LPS (Chapter 7).

Perhaps the situation becomes less confusing if it is considered as a matter of *balance*. The starting material, be it EM or culture supernatant, is a complex mixture of proteins, most with unknown activity. Consequently, the interplay between all these different proteins, including the ones isolated in this study, is also likely to be complex; the result, in terms of host inflammation or host health is the sum of cytokine-inducing and cytokine-inhibiting molecules. The ultimate outcome for the host will depend upon which components are expressed at any given time, and to what extent. The level of expression of the two activities is likely to be controlled by the environment encountered by the bacteria, and an environment that favours the expression of the IL-6-inducing protein, may repress expression of the inhibitor (and *vice versa*). So far, these two activities have only been examined under one growth condition, and it would be interesting to investigate the effect of various environmental factors on the balance of their expression.

An artificial situation has been created by the very act of isolating and purifying these components. In isolating individual components, their activity is no longer seen in context, and it is for this reason that it is important, subsequently, to look at the effects of environmental influences (such as those that might be encountered *in vivo*) on the expression of such proteins. It must be accepted that the isolation and purification of active proteins, such as that described in this study, is merely a starting point. Recently

there has been an upsurge of interest in what has been termed "proteomics" (to complement "genomics"), and this is an area that would lend itself to the study of protein expression in *S. sanguis* under different environmental conditions. Taking things a step further, several groups have developed methods for identifying *in vivo* expressed genes in streptococci, using *in vivo* expression technology (IVET) based methods (Vriesema *et al.* 1997; Kili *et al.* 1999).

8.4 Further work

A number of areas of work have already been mentioned as warranting further investigation, and these will not be further discussed here.

During the initial inflammatory response to bacterial colonisation there will be an influx of immune cells in response to various stimuli. This thesis has focused on the response of some of these cell-types to secreted proteins from *S. sanguis*. However, there are other constituent cell types of the cardiac valves and endocardial tissue that will be in close proximity to the colonising bacteria, most obviously endothelial cells. Cultured human umbilical vein endothelial cells (HUVEC) can be induced to secrete various inflammatory molecules. For example, IL-6 was produced by cultured HUVECs in response to LPS as well as IL-1β, TNFα and IFNγ (Jirik *et al.* 1989), and in response to activated platelets, endothelial cells produced IL-8 by an IL-1-dependent pathway (Kaplanski *et al.* 1993). Endothelial cells of the heart tissues might therefore represent an alternative (or additional) source of inflammatory cytokines in infective endocarditis, and it would be interesting to investigate whether the *S. sanguis* EM (and purified components) induced a cytokine response from such cells. Preliminary studies were carried out using the ECV-304 cell line (ECACC, UK) to assess the ability of EM to induce cytokine secretion from these cells. Unfortunately, the cells were later found to be a bladder epithelial cell line, and so these results have not been included in this thesis.

Another cell type with potential for contributing to inflammatory cytokine secretion are the cardiac myocytes, whose primary function is myocardial contraction. In injuries to

the myocardium where the blood supply to parts of the tissue is disrupted (ischaemic injury), inflammation is an inevitable consequence and involves the recruitment of polymorphonuclear cells (PMN) and monocytes to the injury site. Primary cultures of cardiac myocytes stimulated with LPS, IL-1β or TNFα were demonstrated to express significant quantities of both mRNA and protein for macrophage inflammatory protein-2 (MIP-2: a PMN-specific chemokine) and macrophage chemoattractant protein (MCP: a macrophage-specific chemokine) (Massey *et al.* 1995). Whether cells of myocardial origin would actually contribute to cytokine production in infective endocarditis is doubtful, as they would be located some distance from the vegetation, beneath the endocardial layer. However, these examples serve to illustrate that cells other than the usual range of "immune cells" may be worth investigating for involvement in inflammatory pathology.

The findings in this thesis may also be relevant to the inflammatory pathology of streptococcal septic shock syndrome. In this context, experiments with endothelial cells would be highly relevant, and it may also be interesting to investigate cells such as smooth muscle cells for an ability to be stimulated by *S. sanguis* secreted proteins. Smooth muscle cells (SMC) are present in the walls of blood vessels and may become exposed to platelets or their products as septicaemic inflammation proceeds. SMC have been reported to secrete IL-6 and IL-8 in response to platelets, and this was exacerbated when platelets were pre-activated with thrombin, or when the SMC were pre-stimulated with LPS, TNFα or IL-1α. The cytokine response of the SMC was inhibited by IL-1ra, but not by anti-platelet derived growth factor (PDGF), suggesting that the platelet-induced cytokine production involved IL-1 (Loppnow *et al.* 1998).

Another question that has not yet been addressed is that of the cellular origin of the cytokines produced by PBMC in response to *S. sanguis* EM. To ascertain which cells were responding to the *S. sanguis* EM, various populations could be depleted from the PBMC preparation. For example, immuno-magnetic beads are available which will bind to specific molecules present on the surface of certain populations of cells, and allow their separation from other populations. Purified monocytes can be obtained by

elutriation and it might be useful to repeat the cytokine-inducing experiments on purified monocytes. On the other hand, the use of purified cell populations in such experiments fails to take into account the contribution that may be made to cytokine production by other cells present *in vivo*.

In terms of the induction of a pro-coagulant environment, cultured HUVEC have been reported to express tissue factor (TF), the cell-associated activator of the coagulation cascade, when stimulated with LPS or IL-1 (Colucci *et al.* 1983; Bevilacqua *et al.* 1984). It has now become accepted that cells considered to be non-thrombogenic, such as endothelial cells, may play an active role in inflammatory-associated thrombosis (Rodgers, 1988). Other investigations revealed that TF expression was induced in cultured human cardiac valves by whole *Staph. aureus*, although this was not found to be the case with whole enterococci or viridans streptococci (Drake and Pang, 1988). This might be another area worth investigating: if the secreted proteins from *S. sanguis* were capable of upregulating TF expression on cardiac endothelial cells this might further add to the thrombotic vegetation.

8.5 Final conclusions

This thesis has focused on the cytokine-modulating activities of extracellular proteins from *S. sanguis*, one of the most commonly isolated organisms from cases of infective endocarditis. The unpurified extract (termed exported material, or EM) from blood-grown *S. sanguis* has potent inflammatory cytokine-inducing activities, and some progress has been made toward isolating the active proteins from both EM and secreted culture supernatant protein. In the absence of blood, the material produced by the same organism contains LPS-inhibitory activity which may have relevance to the survival of *S. sanguis* in the oral cavity in the absence of inflammation.

The *S. sanguis* genome is being sequenced, and some of the data that has been reported in this thesis on the physiochemical properties of active proteins could be used to aid in the identification of potential open-reading frames that code for proteins with these activities.

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