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**INFLUENCE OF IRON ON BACTERIAL
INFECTIONS IN LEUKAEMIA.**

by
Jacqueline Marie Yates

Submitted for the degree of Doctor of Philosophy.

The University of Aston in Birmingham
February, 1992.

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THE UNIVERSITY OF ASTON IN BIRMINGHAM.

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SUMMARY

The influence of iron metabolism, both on the invading bacterial pathogen and in the host is widespread and often appears to be crucial in determining the outcome of an infection. This study involved the investigation of leukaemia, a clinical disease where abnormal availability of iron may play a part in predisposing patients to bacterial infection. The iron status throughout a Gram-negative septicaemia and in 20 random, newly diagnosed leukaemic patients was assessed. The results revealed that the majority of the patients exhibited high serum iron levels and serum transferrin saturation often at 100%, with an inability to reduce the latter to within normal values during an infection episode. The antibody response to *P. aeruginosa*, *E. coli* and *K. pneumoniae* outer membrane protein (OMP) antigens were investigated by immunoblotting with sequential serum samples during infection in the leukaemic host. Antibodies to all the major OMPs were observed, although recognition of iron-regulated membrane proteins (IRMPs) was in many cases weak. Results from the enzyme-linked immunosorbent assay indicated that in all patients antibody titre in response to infection was poor.

Sub-MICs of mitomycin C significantly altered the surface characteristics of *P. aeruginosa*. The silver-stained SDS-PAGE gels of proteinase K digested whole cell lysates of strains PAO1, 6750, M7 and PAJ indicated that core LPS was affected in the presence of mitomycin C. In contrast, the rough strain AK1012 showed no observable differences. Results obtained using quantitative gas-liquid chromatographic analysis showed the amount of LPS fatty acids to be unaffected, however, the KDO and carbohydrate content in strains PAO1, 6750 and M7 under Fe⁺ and Fe⁻ growth conditions were decreased by up to 4-fold in the presence of mitomycin C, indicating perturbed expression of LPS. The cell surface became significantly more hydrophobic in the *P. aeruginosa* strains, except AK1012 which was comparatively unaffected.

The induction of protein G (OprG) in *P. aeruginosa* was found to be a sensitive indicator of media iron. The data indicated that expression of OprG can be modulated by growth rate/phase, availability of iron and by the presence of ciprofloxacin in the growth medium.

Key Words: *Pseudomonas aeruginosa*, iron, outer membrane proteins, sub-MICs.

**To my husband Adrian,
for all his love and support
and to my mum and dad and all my family
thank you.**

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ABBREVIATIONS.

AMPS	ammonium persulphate.
ATCC	American Typing Culture Collection.
BIS	N, N' methylene bisacrylamide.
CDM	chemically defined media.
CDM-Fe	iron depleted CDM.
CDM+Fe	iron plentiful CDM.
CF	Cystic fibrosis.
C.F.U.	colony forming unit.
°C	degrees centigrade.
DNA	deoxyribonucleic acid.
EDTA	ethylenediaminetetraacetate.
ELISA	enzyme-linked immunosorbent assay.
GLC	gas liquid chromatography.
h	hour.
IRMPs	iron-regulated membrane proteins.
kDa	Kilodaltons.
KDO	2-Keto-D-manno-2-octulosonic acid.
L	litre.
LPS	lipopolysaccharide.
M	molar concentrations.
MIC	minimum inhibitory concentration.
min	minute.
ml	millilitre.
mg	milligramme.
µg	microgramme.
MOPS	4-morpholino-propane sulphonic acid.
NA	nutrient agar.
NC	nitrocellulose.
NCTC	National Collection of Type Cultures.
OD	optical density.
OD _{470nm}	optical density at 470nm.
OM	outer membrane.
OMP	outer membrane protein.
OprB	outer membrane protein D1.
OprC	outer membrane protein C.
OprD	outer membrane protein D2.
OprE	outer membrane protein E.
OprF	outer membrane protein F.
OprG	outer membrane protein G.

OprH	outer membrane protein H1.
OprI	outer membrane protein I.
OprL	outer membrane protein H2.
OprP	outer membrane protein P.
PBS	phosphate buffered saline.
PIA	Pseudomonas isolation agar.
Sarkosyl	N-lauryl sodium sarcosine.
SDS	sodium dodecyl sulphate.
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis.
TBS	tris buffered saline.
TEMED	N,N,N',N tetramethylethylene diamine.
TIBC	total iron binding capacity.
v/v	volume per volume.
w/v	weight per volume.

1. INTRODUCTION

1.1 THE LEUKAEMIC PATIENT.

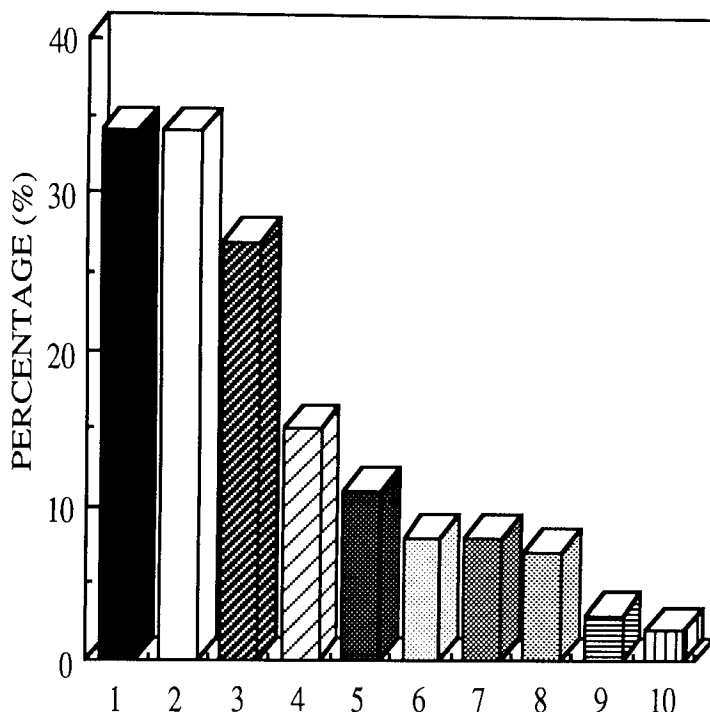
Leukaemia is a cancer of white blood cells which are produced by the bone marrow. It results in uncontrolled proliferation of leukocytes which is reflected in large numbers present in the blood and characteristically involves various organs. Leukaemias are classified on the basis of rapidity of course (acute, subacute and chronic), cell count, cell type and degree of differentiation.

Patients undergoing intensive chemotherapy regimens which employ immunosuppressive drugs are at high risk of becoming infected. Bacterial organisms causing infections in the leukaemic patient are shown (Fig. 1.1; adapted from Bodey *et al.*, 1978). Included under each individual organism are the numbers of patients that had more than one episode of infection caused by the same organism and those organisms cultured from episodes of multiple organism infection. *P. aeruginosa*, *E. coli* and *K. pneumoniae* were the most common organisms causing infection. The rise of infection in neutropenic patients increases substantially if the neutrophil count falls below 1000/mm³ (Bodey *et al.*, 1986). *P. aeruginosa* remains a major source of morbidity and mortality in leukaemic patients, despite improvements in anti-*Pseudomonas* penicillins, carbenicillin, ticarcillin, piperacillin, aminoglycosides and the introduction of the fluoroquinolone ciprofloxacin (Singer *et al.*, 1977; Bodey *et al.*, 1985; 1986; Griffith *et al.*, 1989).

Chemotherapy for leukaemic patients has become increasingly complex. During the last 20 years combination chemotherapy has been shown to be superior to single agent treatment for inducing complete remissions (Crist and Rivera, 1990). Since drug resistance develops in some patients more rapidly than in others, aggressive chemotherapy has been tried to eradicate leukaemic cells before they mutate to a resistant state (Crist and Rivera, 1990). Few new highly effective agents have been developed. Most investigators have focused attention on administering more intensively, via different modes, those agents known to be effective. Induction chemotherapy during the first 2 to 4 months of treatment usually contains 4 drugs, a corticosteroid, vincristine, daunorubicin and asparaginase (Schiffer,

Fig 1.1

ORGANISMS CAUSING INFECTIONS IN PATIENTS WITH LEUKAEMIA



- | | |
|-------------------------|--------------------------|
| 1. <i>E. coli</i> | 6. <i>Proteus</i> |
| 2. <i>Klebsiella</i> | 7. <i>Serratia</i> |
| 3. <i>P. aeruginosa</i> | 8. <i>S. aureus</i> |
| 4. <i>Candida</i> | 9. <i>Bacteriodes</i> |
| 5. <i>Enterobacter</i> | 10. <i>Streptococcus</i> |

1990). The consolidation phase has featured multiple drugs including methotrexate, 6-mercaptopurine and in the past mitomycin C (Crist and Rivera, 1990). Conventional doses of active agents in leukaemia have been 'mixed and matched' in a large number of regimes. Major increases in dosage can be administered during bone marrow transplantation. The availability of haematopoietic growth factors such as granulocyte-colony stimulating factor (G-CSF), may permit more intermediate increases in dose intensity. Several studies have shown that G-CSF can shorten the period of neutropenia during myelosuppressive therapy (Schiffer, 1990). This could have a major impact on the acquisition and severity of infections, the incidence of which increases with the duration of myelosuppression.

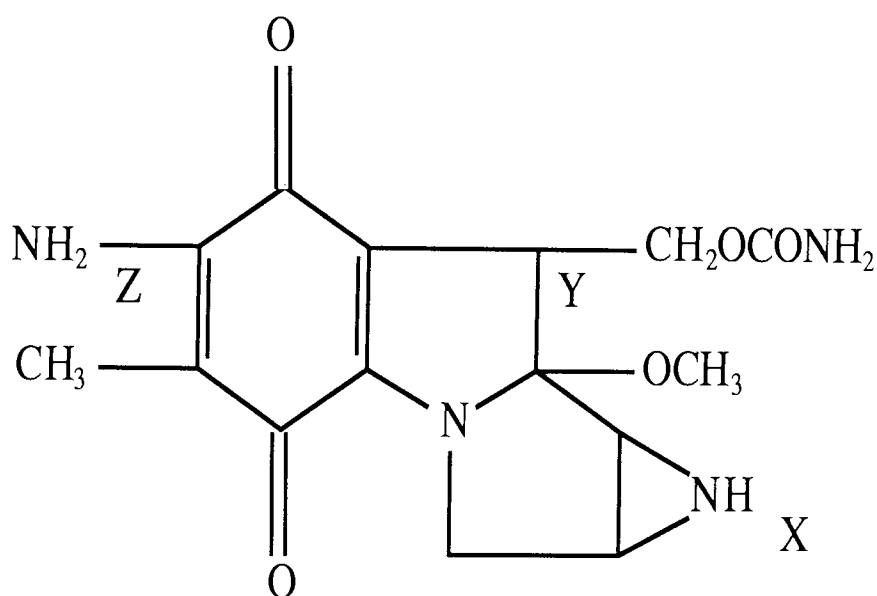
1.1.1 Mechanism of action of mitomycin C.

Mitomycin C, isolated from *Streptomyces caespitosus* has a molecular weight of 334. It possess significant antimicrobial as well as antitumour activity (Hamilton-Miller, 1984; Doll *et al.*, 1985). Mitomycin C is an alkylating agent which covalently binds to DNA with three potential centres of activity, a dihydroquinone moiety, an aziridine ring and a C-10 carbamate group (Fig 1.1.1; Pratt and Ruddon, 1979). For antitumour activity the carbamate must be reduced and the methoxy group lost. A number of general points have been found:

- 1) The aziridine ring is not essential for antibacterial activity, but it is for antineoplastic activity.
- 2) Increasing water solubility and decreasing lipophilic properties results in enhanced antitumour activity.
- 3) Decreased binding to proteins results in enhanced activity.
- 4) Substitutions on positions X, Y and Z have significant effects on activity (Crooke, 1979).

Mitomycin C selectively inhibits DNA synthesis in both bacteria and tumour cells by DNA alkylation and interstrand and intrastrand cross linkage. Maximum effect is achieved during late G₁ and S phases of the cell cycle (Reynolds, 1989). The O⁶ positions of guanine residues in DNA is considered to be the most likely place of attachment. The aziridine ring is known to be involved in cross linking suggesting that this is critical for killing of mammalian cells (Pratt and Ruddon, 1979). However, mitomycin C analogs lacking the aziridine ring inhibit DNA synthesis and cell division in bacteria, with loss of viability. These aziridine-deficient analogs bind monofunctionally, but do not cross-link the DNA. Therefore, in bacteria at least, monofunctional alkylation is clearly damaging (Pratt and Ruddon, 1979). After enzymatic reduction of the antibiotic quinone moiety, the tertiary methoxy group is eliminated and the carbonium ion generated at position X can react with a DNA guanine. A second carbonium ion generated at position Y can then react with the guanine in a second DNA strand to form cross-links between DNA strands and preventing replication (Pratt and Ruddon, 1979; Hoey *et al.*, 1988).

Fig 1.1.1



Structure of mitomycin C.

Although possessing antibacterial activity, mitomycin C is used exclusively for the treatment of malignant neoplasms. It is routinely given intravenously (I.V.), although it is used intra-arterially, intra-pleurally and via instillation into the bladder. After injection of 30mg, 20mg or 10mg I.V., the maximum serum concentrations are $2.4\mu\text{g}\cdot\text{ml}^{-1}$, $1.7\mu\text{g}\cdot\text{ml}^{-1}$ and $0.5\mu\text{g}\cdot\text{ml}^{-1}$, respectively (Reynolds, 1989). Clearance is effected primarily by metabolism in the liver, but metabolism occurs in other tissues such as the kidney and spleen. It has been used successfully in a wide range of neoplastic conditions including carcinomas of stomach, breast, bladder and hepatic cancer, acute and chronic leukaemias and finally Hodgkin's disease (Reynolds, 1989). In general, mitomycin C is given I.V. at intervals of 1-6 weeks depending on whether other drugs are given in combination and on the recovery of the bone marrow. The principal dose-limiting toxicity of mitomycin C is delayed cumulative myelosuppression occurring 3-4 weeks after drug administration with recovery generally within 8 weeks and manifested primarily as leukopenia and thrombocytopenia (Pratt and Ruddon, 1979). The most common general side effects are fever, anaemia, anorexia, nausea and vomiting (Crooke, 1979; Doll *et al.*, 1985).

The mechanism of interaction between antimicrobial agents and antineoplastic agents against

bacteria has not well been studied. It has been noted that compounds which inhibit protein synthesis were found to enhance mitomycin C-induced DNA breakdown in *E. coli* (Yamashiro *et al.*, 1986). Mitomycin C has been found to be synergistic with the β -lactams and aminoglycosides against *P. aeruginosa*, *E. coli* and *K. pneumoniae* (Yamashiro *et al.*, 1986).

1.2 THE HOST-BACTERIUM INTERACTION.

Better understanding of the leukaemic host environment encountered by bacterial pathogens and their adaptation to such conditions are leading not only to a radically different view of the behaviour of pathogens *in vivo*, but also to ideas concerning the development of new antibacterial agents.

There has been a growing interest in the mechanism whereby rapidly-dividing cells, especially malignant cells, obtain sufficient iron to support their multiplication. Indeed, it is only recently that the critical role of iron in the growth of such cells has been generally appreciated (Brock and Maniou-Fowler, 1983; Taetle *et al.*, 1985; Griffiths and Bullen, 1987). During clinical disease, host iron metabolism may alter and infection of a compromised patient is likely to differ significantly from that of a healthy host. There is evidence that some of the serum proteins that bind iron compounds can be reduced significantly in certain clinical conditions. For example, a reduction in the level of transferrin in leukaemia (Hunter *et al.*, 1984a) and in kwashiorkor (McFarlane *et al.*, 1970) has been noted. In these conditions there is an increased incidence of infection (Griffiths and Bullen, 1987). The possibility that iron may be made more readily available to some pathogens by an increase in the degree of transferrin saturation, causing enhanced incidence of infection in the leukaemic patient is considered in this study.

1.2.1 Infection and the host environment.

One common and essential factor in all infections is the ability of the invading pathogen to multiply successfully in the tissues of the host. Here the bacteria must produce the full complement of virulence determinants required for pathogenicity. The cardinal requirements

for pathogenicity are to enter the host, multiply in the host, resist or not stimulate host defences and to damage the host (Smith, 1990). In most cases these properties are influenced greatly by the nature of the bacterial surface. Most investigations into bacterial virulence have been carried out with organisms grown *in vitro* under conditions that do not necessarily reflect microbial behaviour *in vivo*. It is well known that bacteria can alter their metabolism rapidly in response to environmental changes and that they are capable of existing in a variety of physiological states that can be quite different from one another. In general, little is known about the alterations that can occur in pathogenic bacteria as they adapt to and multiply in the environment found in host tissues under bacterial attack. These are not only poorly defined but also likely to change as an infection proceeds. The goal of studies on pathogenicity is to identify the determinants of these requirements and to relate their chemical structure to biological function. Part of achieving this goal is to show that the determinant is either present or produced *in vivo* (Brown and Williams, 1985b).

Perhaps one of the best understood aspects of the host environment concerns the availability of iron. Most of the body's iron is found intracellularly, in ferritin, haemosiderin or haem and that which is extracellular in body fluids is attached to the high affinity iron-binding glycoproteins transferrin and lactoferrin (Griffiths, 1991). Transferrin is found mainly in serum, whereas lactoferrin is found in neutrophils and in external secretions (table 1.2.1; adapted from Griffiths, 1987b).

Since these iron-binding proteins are usually only partially saturated with iron and have association constants of approximately 10^{36} , the amount of free iron in equilibrium with these proteins is about 10^{-18}M . Thus, although there is normally an abundance of iron present in body fluids, the amount of free iron is far too small to sustain bacterial growth (Bullen, *et al.*, 1978; Bullen, 1981). In addition, during infection the host reduces the total amount of iron bound to serum transferrin and this decrease is called the hypoferraemia of infection. Although the triggering signal appears to be due to the leukocyte endogenous mediator (LEM), the subsequent processes which lead to the reduction of serum iron levels are not fully understood (Kluger and Bullen, 1987; see section 1.5.3).

Table 1.2.1 Distribution of host iron-binding glycoproteins.

Lactoferrin	Saliva Tears Nasal secretions Intraocular fluids Seminal fluids Cervical mucus Colostrum Milk (human)
Transferrin	Polymorphonuclear leukocytes Serum Lymph

1.2.2 Iron-binding proteins and host defence.

The multiplication of bacteria in the largely undefined and changing environment of host tissues is an essential feature of any infection and an ability to interfere with this process plays a major role in host resistance to infection.

The transferrins comprise a group of closely related proteins, the best known of these are serum transferrin and lactoferrin. These iron-binding glycoproteins are structurally similar, consisting of a single polypeptide chain with a molecular weight of about 80,000 (Brock, 1989b). This chain is folded to give two largely independent and similar, but not identical globular domains, each of which can bind one ferric ion (Brock and Maniou-Fowler, 1983). Binding of iron requires the synergistic binding of an anion, which under physiological conditions is either carbonate or bicarbonate (Brock and Maniou-Fowler, 1983). A number of functions have been proposed for the transferrins, many of which are still controversial, particularly in the case of lactoferrin.

1) Serum transferrin functions as the major iron transport protein and is an essential requirement for cellular proliferation (Vostrejs *et al.*, 1988). Cells which require iron express a receptor for transferrin, which is a transmembrane glycoprotein consisting of two identical disulphide-bonded 90 kDa subunits (Brock, 1989b). Expression of transferrin

receptors is linked to the iron requirements of the cell and appears to be a reliable marker for proliferating cells and the number of receptors is an indication of the rate of proliferation of both normal and malignant cells (Hunter *et al.*, 1984a).

2) Numerous studies have confirmed the ability of transferrins to impede the *in vitro* growth of iron-requiring bacteria and this effect is blocked if the proteins are saturated with iron (Bullen, 1981; Ellison *et al.*, 1988; Brock, 1989b). Iron transport systems appears to be an essential virulence determinant for many infectious bacteria. In response to iron-restricted growth conditions several pathogens produce siderophores. These can remove iron from the host iron-binding proteins in conjunction with the expression of a cognate receptor on the bacterial membrane, involved in transporting the ferric-siderophore complex into the cell (Redhead and Hill, 1991). Microbial growth therefore, will not occur until sufficient siderophore is available to compete effectively with transferrin and lactoferrin for bound iron. This results in a considerable extension of the lag phase, as exemplified by Brock (1989b), who showed that an enteropathogenic strain of *E. coli* inoculated into human milk, which contains lactoferrin, did not start to grow for about 10h. Addition of excess iron reduced the lag period to 3h, while addition of the enterobacterial siderophore enterobactin virtually abolished the lag phase. Administration of inorganic iron to animals, sufficient to saturate transferrin have been shown repeatedly to potentiate bacterial and fungal infections (Murray *et al.*, 1978). The major hypothesis advanced for this activity has been that transferrins produce an iron-deficient environment that limits bacterial growth. However, the wealth of *in vitro* observations need to be applied with caution to the situation *in vivo*, which is far more complex than a simple competition between transferrins and siderophores, as iron may be potentially available from other sources (Brock, 1989b). Therefore, it is proposed that serum transferrin provides a degree of non-specific immunity to bacterial infection. Lactoferrin may have an additional antimicrobial role as part of the bactericidal armoury of neutrophils (see section 1.5.3).

3) While the role of transferrin as an iron donor to cells is now well established (Hunter *et al.*, 1984a), the existence of a similar role for lactoferrin remains doubtful (Brock, 1989b). Lactoferrin may act principally as a scavenger of iron in conditions where transferrin is unable to bind iron well, such as low pH (Griffiths, 1987b).

4) Transferrin plays an essential role in erythropoiesis by shuttling iron from the storage sites in the liver and reticuloendothelial system to the erythroid precursors in the bone

marrow. Transferrin levels have been used widely in the evaluation of anaemia (Hunter *et al.*, 1984a).

5) The transferrin levels fall with malnutrition and rise with appropriate nutritional therapy more rapidly than most other indicators. Low levels of transferrin and/or a failure to rise with nutritional therapy are poor prognostic signs in children with kwashiorkor and adults with cancer and other serious diseases (McFarlane *et al.*, 1970).

These data suggest that the transferrins play a key role in several physiologic processes that can become deranged in disease. It has become clear that transferrin plays a crucial role in cell division and in particular lymphocyte transformation (Brock and Maniou-Fowler, 1983). The expansion of clones of lymphocytes, an essential feature of immune responses, involves increased cellular metabolic activity and cell division, and hence *de-novo* synthesis of some iron-containing compounds and an increased iron requirement (Brock and Maniou-Fowler, 1983). It is therefore possible that an inadequate delivery of iron might prevent lymphocyte transformation from proceeding optimally. Evidence to support this contention, albeit indirectly, comes from several studies in human subjects and in experimental animals, which report that iron deficiency can result in impaired cell-mediated immune responses (Brock and Maniou-Fowler, 1986; Kuvibidila, 1987).

1.2.3 Iron and the antibacterial function of polymorphonuclear leukocytes.

In vivo, the effectiveness of phagocytosis depends on several factors including, the ratio of bacteria to phagocytic cells, the rate of ingestion of the bacteria, and the rate of destruction or growth of the bacteria. Polymorphs are found in plasma and inflammatory exudates, both of which contain unsaturated iron-binding proteins. The influence of these proteins in combination with other antibacterial factors, can be of critical importance. This is illustrated by the fate of *P. aeruginosa* in experimental intraperitoneal infections in rabbits (Bullen *et al.*, 1974; 1976). The introduction of *P. aeruginosa* produced a massive exudate of polymorphs, but the response was the same both in lethal and non-lethal infections, with or without the addition of iron compounds. In all cases there was comparatively little change in the mononuclear cell population (Bullen *et al.*, 1974). Infection produced a sharp fall in the percentage saturation of transferrin of both the plasma and the peritoneal fluid. In both

lethal and non-lethal infections the majority of the viable bacteria were extracellular suggesting that the internal bactericidal mechanisms of the polymorphs were highly efficient (Bullen *et al.*, 1974). This was confirmed by experiments with ^{32}P - and ^{14}C -labelled bacteria where only a small percentage of intracellular organisms were viable (Bullen *et al.*, 1976). Since the number of polymorphs was the same in both lethal and non-lethal infections, the rate of extracellular growth and the rate of phagocytosis determined the outcome of the infection.

In non-lethal infections all the evidence suggests that unsaturated iron-binding proteins make a large contribution to resistance. When sufficient Fe^{3+} was administered to saturate serum transferrin, bacteria normally producing a non-lethal infection became lethal. Instead of declining in numbers the extracellular bacteria increased rapidly until death occurred. *In vitro*, it was found that saturation of the iron-binding capacity of the peritoneal fluid allowed the bacteria to grow twice as quickly as before, with a generation time of 34min. Assuming that this also occurred *in vivo* the increase in the speed of extracellular growth appeared to be crucial and was sufficient to overwhelm the polymorphs. The importance of iron-binding proteins in resistance to *P. aeruginosa* is also shown by the increase in susceptibility that occurs when the iron-binding capacity of plasma transferrin is saturated. In rabbits, the lethal dose (LD) was reduced by 100-fold (Bullen *et al.*, 1974) and in mice the LD 50 by 1,000-fold (Forsberg and Bullen, 1972). These experiments provide additional evidence that an increase in the availability of iron, which destroys the bactericidal or bacteriostatic properties of plasma, has an adverse effect on phagocytic efficiency and can lead to polymorphs being overwhelmed by the bacteria. Thus, the role of transferrin in extracellular fluid may be of critical importance in deciding the outcome of these infections.

1.2.4 Clinical consequences of excess iron.

One of the main sources of iron for bacterial growth is Fe^{3+} bound to transferrin or lactoferrin. The normal level of saturation for these proteins is about 30% and any condition which fully saturates the iron-binding proteins, potentially makes Fe^{3+} freely available to bacteria. Even an increase in saturation may make it more easily attainable to some organisms. Thus, human serum with 50% transferrin saturation had a bacteriostatic

effect for 18h against *Candida albicans* which was abolished by raising the saturation to 90%. A further increase to 120% resulted in an increase in fungal growth by about 30-fold (Caroline *et al.*, 1969). Several instances are recorded where excess iron has been made available accidentally, or as a consequence of treatment, which resulted in enhancement of infection (Murray, 1978; Melby *et al.*, 1982; Ward *et al.*, 1986). Despite hypoferraemia being one of the most common findings of chronic disease, leukaemia appears to be the exception (Kluger and Bullen, 1987). Several investigators have shown leukaemic patients to have high serum iron levels, low levels of serum transferrin which is fully saturated with iron. In addition these patients were found to be unusually susceptible to infections (Caroline *et al.*, 1969; Estey *et al.*, 1982; Hunter *et al.*, 1984a; 1984b). These results suggest that the leukaemic host may constitute an iron rich environment, in sharp contrast to the iron-restricted conditions found in patients with cystic fibrosis, burns and urinary tract infections (Brown *et al.*, 1984; Shand *et al.*, 1985; Ward *et al.*, 1988).

In attempting to summarise the clinical consequences of increased availability of iron there are a number of points which should be emphasised. Firstly, raised levels of freely available iron, as a result of fully saturated transferrin, low serum transferrin and high serum iron levels in humans, increases the likelihood of infection. This suggestion is strongly supported by experimental evidence and although much of the clinical evidence is circumstantial, it is quite consistent and increasingly convincing. Secondly, the administration of iron-chelating agents like desferrioxamine, which can transport iron to some bacteria, can be dangerous, especially in patients already overloaded with iron (Kluger and Bullen, 1987).

1.2.5 Environment of leukaemic host.

Many abnormalities in immunological function have been documented during the course of malignant disease in patients with a variety of different cancers. There is, however, considerable controversy whether such abnormalities are the result of cancer or represent an important causal factor. Haskell (1977) suggests several points of view. The concept of 'immunologic surveillance' was postulated, where the immune response is the principal defence against neoplastic cells. It is reasoned that the immune response destroyed cancer

cells while they were still in the incipient stages of tumour formation. This theory predicts that impaired immunity would inevitably increase susceptibility to cancer and suggests that immunity against a variety of neoplasms would exist in all normal adults. However, other studies have failed to demonstrate enhanced carcinogenesis with immunosuppressive treatment (Haskell, 1977). In a critical review of the theory of immunologic surveillance, Schwartz (1975) proposed that defective immunity may lead to cancer, not through surveillance against neoplastic cells, but by the failure to terminate lymphoproliferation triggered by an antigen. The author concluded that it may be unrealistic to expect the enormous complexities of cancer to crystallise within the confines of a single theory. Some neoplasms may well arise as a result of spontaneous mutation, while others may be induced by viruses, chemical carcinogens, X-rays, or hormonal stimulation. Some may be eliminated by an immune response, whereas others may actually require an immune response for their development. Even if one believes that the immune system plays no role in the aetiology of cancer, it clearly is an important host defence mechanism for many of the complications that occur during treatment, specifically infections. As mentioned previously, there is strong evidence that bacteria grow under iron-depleted conditions, however, the leukaemic host may be the exception representing an iron rich environment. Hyperferraemia appears to be a common finding in leukaemic patients represented by highly saturated transferrin and may consequently predispose them to infection.

There is a paucity of information about the impact of combination chemotherapy on the immune system. The major studies have taken place in leukaemic patients where a number of investigators have analysed the impact of various programmes of intensive combination chemotherapy, on long-term survival and immunologic function. Leventhal *et al.*, (1974) concluded that the majority of patients with acute leukaemia showed depressed B-cell function during combination chemotherapy. There was also a correlation between a poor prognosis and depressed T-cell function during the induction phase of chemotherapy. The authors postulated that this poor survival was probably due, at least in part, to an increased incidence of fatal infections.

1.3 INFLUENCE OF THE *IN VITRO* AND *IN VIVO* ENVIRONMENT ON THE SURFACE PROPERTIES OF GRAM-NEGATIVE BACTERIA.

The prevailing environment exerts a profound influence on the properties of bacteria especially in relation to specific nutrient deprivation (Brown and Williams, 1985a; 1985b; Dalhoff, 1985), the rate of growth (Brown *et al.*, 1988a; 1990; Smith, 1990) and to their mode of growth as mobile planktonic cells or glycocalyx-enclosed adherent microcolonies (Costerton *et al.*, 1983; 1987; Brown and Williams, 1985b; Costerton and Lappin-Scott, 1989). The ability of a microorganism to modify and adapt to its surroundings is clearly advantageous in terms of survival and may be important in determining bacterial pathogenicity (Costerton *et al.*, 1979).

It is well recognised that the amount of iron normally available to bacteria in the tissue fluids of human and animals is extremely small. This iron-restricted environment induces phenotypic changes both in the metabolism and in the composition of the outer membrane of bacteria growing *in vivo* (Bullen, 1981; Griffiths, 1983a; 1983b; Brown and Williams, 1985b). Growth of bacterial pathogens in the presence of partly saturated iron-binding glycoproteins can lead to the synthesis and secretion of siderophores, to the production of new outer membrane proteins and to changes in transfer RNAs (Griffiths, 1983a; 1983b). Such alterations are considered necessary features of cellular economy during the stress of iron-restricted growth and some phenotypic changes are known to be essential for the acquisition of iron by bacteria under these environmental conditions. Bacteria which cannot sequester iron *in vivo* are unable to proliferate.

The structural flexibility of the bacterial envelope, therefore, is important in understanding the host-bacterium interaction in infection, since phenotypically-induced alterations in the surface components of a bacterium will affect its virulence and immunogenicity and in addition, its sensitivity to antibiotic therapy (Costerton *et al.*, 1979; Brown and Williams, 1985a; 1985b; Smith, 1990). The surface properties of bacteria *in vivo* are in turn likely to be influenced by host defence mechanisms and the presence of sub-MICs of antibiotics (Finch and Brown, 1975; Brown *et al.*, 1979; Anwar *et al.*, 1983a; Brown and Williams, 1985b; Dalhoff, 1985), hence influencing the capacity of the bacteria to cause disease.

Bacteria may progressively lose virulence on subculture *in vitro*, which is restored by animal passage (Forsberg and Bullen, 1972; Smith, 1977). Resistance to serum killing and phagocytosis has also been shown to increase following growth *in vivo* (Finn *et al.*, 1982). This may be a result of selection and/or phenotypic variation.

The environment for bacterial pathogens during infection is complex and constantly changing. It is different from that used *in vitro* for most experiments on pathogenicity (Smith, 1990). Hence, when pathogens are moved from one type of environment to another, selection and phenotypic change occur, which can have implications for studies on pathogenicity. First, putative determinants of pathogenicity, indicated by experiments using *in-vitro*-grown organisms, may not be produced *in vivo*. Second, and perhaps more importantly, virulence determinants found *in vivo* may be missed because they are not formed under arbitrarily chosen growth conditions *in vitro*. Nor is the consequence of such environmental change confined to determinants of pathogenicity. Killed vaccines prepared from *in-vitro*-grown organisms or their products may be incomplete with respect to immunising antigens, which are produced *in vivo* either by infection or by live attenuated vaccines (Brown *et al.*, 1988b; Smith, 1990). In addition, drugs, adequate in tests against *in-vitro*-grown bacteria, may not be effective against pathogens *in vivo* (Brown, 1977; Brown and Williams, 1985b).

Direct electron microscopic examination has revealed that the adherent surface associated microcolony mode of growth is an important feature in the growth of many bacteria *in vivo*, especially in chronic infections (Costerton *et al.*, 1983; 1987). The microcolony mode of growth is perhaps best exemplified by *P. aeruginosa* growing in the lungs of CF patients with chronic infection (Lam *et al.*, 1980). Although electron microscope studies produce information about the ultrastructure of bacteria growing *in vivo* on surfaces or otherwise, little is known of the general biochemistry, envelope composition or resistance to host defences and antibiotics. Therefore, to improve our knowledge of *in vivo* bacteria, *in situ*, cells need to be harvested directly. Several investigators have studied the properties of *in vivo* grown cells by using chamber implants (Finn *et al.*, 1982; Cochrane *et al.*, 1988), which have the advantage of localising the infection and simplifying the procedures necessary to recover the bacteria. However, they do not represent the complete *in vivo*

situation because, whilst, soluble humoral factors and antibiotics may penetrate, cellular antimicrobial defences are excluded (Brown and Williams, 1985b). Neutropenic animals have been used to increase yields of bacteria and to ease purification (Cryz *et al.*, 1983; Kadurugamuwa, 1985; Kadurugamuwa *et al.*, 1988), which have proved useful to mimic infections particularly in immunosuppressed patients such as in leukaemia. Furthermore, if *in vitro* grown cells are used as an inoculum, the organisms will not exhibit *in vivo* surface characteristics for several generations (Williams *et al.*, 1984). Finally, non-adhering bacteria are typically studied and these cells would exhibit properties vastly different from those of corresponding sessile population of cells attached to surfaces (Costerton *et al.*, 1983; 1987).

Nevertheless, such studies have yielded important evidence of the differing characteristics of *in vitro* and *in vivo* bacteria. A few investigators have succeeded in studying bacteria *in situ* in animal infections without the use of implants (Griffiths *et al.*, 1983; Sciortino and Finkelstein, 1983). More importantly *P. aeruginosa* and several other species of Gram-negative bacteria have been recovered directly from human CF lung infection (Anwar *et al.*, 1984; Brown *et al.*, 1984; Cochrane *et al.*, 1988), urinary tract infections (Lam *et al.*, 1984; Shand *et al.*, 1985) and human burn wound infection (Ward *et al.*, 1988). Finally, the phenotypic changes in the OM in response to growth in iron-depleted media have been studied in a variety of infections by examining the human immune response both to protein (OMP) and LPS components (Anwar *et al.*, 1985; Shand *et al.*, 1988b; 1991; Smith and Alpar, 1991). The effects of specific nutrient limitation, growth rate and sub-MICs of antibiotics on the surface properties of bacteria will be illustrated, including their possible relevance to the *in vivo* situation.

1.3.1 Nutrient availability.

1.3.1.1 Response to iron deprivation.

Iron is essential for many bacterial enzymes including those involved in the electron transport system, the flavoproteins and oxygen metabolism (Griffiths, 1987a). In *E. coli* the process of DNA replication is known to be dependent on iron. Iron-containing

ribonucleotide reductases catalyse the enzymatic conversion of ribonucleotides to the deoxy-ribonucleotides, which is an obligatory step in DNA synthesis. Therefore, iron appears to be an essential element for all forms of life (Griffiths, 1987a), except lactobacilli, which seem to utilise vitamin B12 co-enzyme as a factor for the reduction of ribonucleoside triphosphate (Archibald, 1983). In spite of the virtual absence of freely available iron in normal body fluids, and of the hypoferraemia of infection, pathogenic bacteria can multiply successfully *in vivo* to establish an infection. Bacterial pathogens must, initially, be able to adapt to the iron restricted environment usually found *in vivo* and develop mechanisms for acquiring iron. Griffiths, (1987b) has outlined 4 mechanisms by which bacteria might be expected to assimilate protein bound iron or acquire it from liberated haem in the body. These are:

- 1) Through proteolytic cleavage of the iron-binding glycoprotein, leading to disruption and release of iron from the iron-binding site.
- 2) By reduction of the Fe^{3+} complex and the consequent release of Fe^{2+} from the glycoprotein.
- 3) Through direct interaction between receptors on the bacterial cell surface and the Fe^{3+} glycoprotein complex.
- 4) By producing low molecular weight iron-chelating compounds, called siderophores, that are able to remove iron from the Fe^{3+} -glycoprotein complex and to deliver the iron to the bacterial cell (Neilands, 1981b; 1984).

Many microorganisms combat the low availability of iron in aerobic environments by producing siderophores (Neilands, 1984) which convert polymeric ferric oxyhydroxides, present in aerobic environments, into soluble chelates which are substrates for high affinity bacterial transport mechanisms (Sriyosachati and Cox, 1986). The ferric-siderophore complex may then be internalised into the bacterial cell when it binds to a cognate IRMP receptor on the bacterial membrane. In *P. aeruginosa* once the complex is transported across the membrane, the Fe^{3+} is reduced to Fe^{2+} by the action of ferripyoverdin reductase and the Fe^{2+} having a low affinity for pyoverdin, is released from the complex. These low molecular weight chelators are part of what is termed the 'high-affinity' iron transport system. A low affinity transport system is thought to operate when iron is freely available, but little is known about the mechanisms involved. The most compelling evidence for the

presence of a low affinity system comes from mutational analysis. All vestiges of the 'high affinity' system, can be removed without impairing growth rate provided higher levels of iron are available (Neilands, 1981b).

A number of siderophores have been described, the majority of which are phenolic (catechols), or hydroxamic acid compounds (Martinez *et al.*, 1990). Enterobactin from *Escherichia*, *Klebsiella* and *Salmonella* species has been well-characterised. This molecule, a cyclic triester of 2, 3-dihydroxy-N-benzoyl serine, is synthesised under conditions of iron deprivation and removes iron from the iron-binding proteins and promotes bacterial growth (Griffiths, 1983b; Neilands, 1984). The most outstanding feature of this siderophore is its extremely high affinity constant of 10^{52} for ferric iron at neutral pH (Neilands, 1981a). However, the chelator is used only once for transporting iron into the bacterial cell, since the cyclic triester linkages of Fe³⁺-enterobactin require cleavage by a specific esterase to release iron (Griffiths, 1987b). Organisms which secrete this siderophore are therefore capable of competing efficiently with host glycoproteins for iron. Many strains of *E. coli* capable of causing general extra-intestinal infection in man carry the Colicin V plasmid (ColV) and synthesise a plasmid-encoded hydroxamate type siderophore called aerobactin in addition to enterobactin (Warner *et al.*, 1981). Aerobactin is possibly inferior to enterobactin in respect of its affinity constant (10^{23}), however, it can be recycled following simple reduction of Fe³⁺ to Fe²⁺ within the cell and thus is an energetically less expensive way of assimilating iron (Griffiths, 1987b). Furthermore, serum antibodies to enterobactin have been demonstrated *in vivo*, which could reduce the effectiveness of iron uptake by this siderophore, so favouring the aerobactin system (Moore *et al.*, 1980). This, coupled with the possibility that enterobactin binds to serum albumin (as do other phenolate siderophores), thus rendering them inactive may explain the need to produce an alternative siderophore (aerobactin) when iron is limited.

Although enterobactin-mediated iron uptake appears to be the main endogenous system used by *Escherichia coli*, *Klebsiella* and *Salmonella* species for accumulating iron, these organisms also utilise a variety of hydroxamate-type siderophores including ferrichrome and ferrioxamine B, which are not synthesised by the organisms themselves but produced by other microorganisms (Leong and Neilands, 1976; Neilands, 1981b; 1984). In addition,

certain strains of *E. coli* possess a citrate-mediated iron-transport system, induced when grown *in vitro* in an iron-limited media containing citrate (Hancock *et al.*, 1976).

Pseudomonas species are known to produce several siderophores during iron restriction, ferribactin, ferrioxamines, pyoverdin, pyochelin and pseudobactin (Cox, 1980; Neilands, 1982; 1984). Of these, pyochelin and pyoverdin are of most interest in infection. Although pyoverdin, which has a binding constant for iron of 10^{32} (Griffiths, 1987b), has been shown to promote the growth of *Pseudomonas* in the presence of transferrin (Cox and Adams, 1985), pyoverdin-negative strains have been isolated from human infections. In contrast, no pyochelin (binding constant 10^5) negative strains have been found. However, this siderophore does have dramatic effects on the virulence of *P. aeruginosa* and it has been shown to promote experimental infection in mice (Cox, 1982). The iron uptake of ferri-pyochelin by *P. aeruginosa* involves two stages, an energy-independent step, presumably involving binding to the cell surface, followed by an energy-dependent process (Cox, 1980). Furthermore, although there has been considerable interest in pyochelin as a siderophore, recent work suggests that the synthesis of pyoverdin may be more important for the growth of *P. aeruginosa* in serum (Ankenbauer *et al.*, 1985; Morris and Brown, 1988). Pyochelin is, apparently, synthesised very poorly by bacteria grown in serum offering little benefit to the organism (Ankenbauer *et al.*, 1985). Recently, Haas *et al.*, (1991) have demonstrated that *P. aeruginosa* in the CF lung is iron stressed and responds by synthesising pyoverdin which subsequently binds ferric iron.

An essential part of high-affinity iron-uptake systems based on siderophores is the production of OMP receptors (Neilands, 1981b). Specific receptors are required for iron-siderophore complexes, since their molecular weight exceeds the exclusion limit of the small water-filled porin channels which are present in the OM of Gram-negative bacteria (Nikaido and Nakae, 1979). The IRMPs of *E. coli* have been extensively studied and are designated according to their molecular weight as 74, 74.5, 76, 78, 81 kDa proteins (Neilands, 1982; Griffiths, 1983b). The 81 kDa FepA protein, product of the *fepA* gene is the receptor for Fe^{3+} -enterobactin. The 78 kDa FhuA protein, product of the *fhuA* gene is the receptor for ferrichrome, while the 76 kDa *fhuE* protein is the receptor for coprogen neither of which is produced by *E. coli*. The IutA (74.5 kDa protein) is the ColV-plasmid-encoding receptor

for aerobactin (Griffiths, 1987b). Finally, the 74 kDa protein expressed only by strains lacking cir protein, is a possible receptor for an as yet unknown siderophore.

Under conditions of iron limitation, expression of 3-9 extra high molecular weight proteins (70 to 100 kDa) are induced in the OM of a wide range of Gram-negative bacteria (Klebba *et al.*, 1982; Williams *et al.*, 1984; Hancock *et al.*, 1990). Studies with mutants lacking individual IRMPs have shown that they function as receptors for the binding of complexes of iron with siderophores (Neilands, 1982; Cody and Gross, 1987; Hancock, 1990). These siderophores are either produced by the same bacterium under iron starvation conditions, or can be supplied exogenously by other sources. *P. aeruginosa* can produce at least two classes of siderophores as well as two or more IRMPs (Mizuno and Kageyama, 1978; Ohkawa *et al.*, 1980; Sokol and Woods, 1983; Brown *et al.*, 1984).

Studies on *P. aeruginosa* strains isolated from human infections provide evidence that bacteria grow under conditions of iron deprivation *in vivo*, in both chronic (Brown *et al.*, 1984; Cochrane *et al.*, 1988) and acute infections (Shand *et al.*, 1985; Ward *et al.*, 1988). However, isolation of bacteria directly from a leukaemic host has not yet been achieved. Brown *et al.*, (1984) showed that mucoid *P. aeruginosa* in the cystic fibrosis lung grows under iron-restricted conditions. Indeed, their work gave the first direct biochemical evidence of the physiological state of bacteria when infecting humans. The OMP composition of mucoid *P. aeruginosa* recovered without subculture from the sputum of a cystic fibrosis patient was shown to contain three iron-regulated outer membrane proteins with molecular weights in the range 80-90 kDa. These iron-regulated proteins were shown to be antigenic and recognised by antibodies in the patient's serum (Anwar *et al.*, 1984).

P. aeruginosa PAO1 can be shown to express three or four IRMPs in the range of 75 to 90 kDa. Several authors have suggested that there may be some specificity between ferripyoverdin molecules and their cognate IRMP receptor, perhaps attributable to variation in the peptide backbone of the pyoverdin molecule. Poole *et al.*, (1991) demonstrated greatly reduced ferripyoverdin uptake in a mutant lacking a 90 kDa IRMP. The residual uptake capacity of the mutant suggested that there may be multiple uptake pathways. Meyer *et al.*, (1990) showed that polyclonal antiserum raised against an 80 kDa IRMP could reduce ferri-

pyoverdinin uptake in some, but not all, *P. aeruginosa* strains tested. Similarly, Smith *et al.*, (1991) found that a monoclonal Ab raised against an 85 kDa IRMP reacted only with approximately one third of all strains tested. These data suggest that there must be very subtle differences in the high molecular weight IRMPs for them to be functionally related and yet immunologically distinct. Further analysis awaits nucleotide sequence determination. Interestingly, Heinrichs *et al.*, (1991) have shown that a 75 kDa IRMP is a ferripyochelin receptor which became over-expressed in a ferripyoverdin-deficient mutant. Complexation of the purified protein with ferripyoverdin protected it from trypsin digestion. Maximal uptake via this receptor was seen only after early stationary phase had been reached, perhaps explaining why the Calgary group (Sokol and Woods) had not observed it with their uptake studies using logarithmic phase cells. Sokol and Woods (1983) and Sokol (1984) have shown that ferripyochelin binds, *in vitro*, to a protein with a molecular weight of 14 kDa. This low molecular weight protein is also expressed in greater amounts in cells grown under iron limitation. These workers suggest that the high molecular weight membrane proteins induced by iron limitation are not involved in the binding of ferripyochelin to the bacterial cell. However, the induction of a low molecular weight 14 kDa IRMP (the receptor for ferripyochelin) under conditions of iron-depletion (Sokol and Woods, 1983; 1984; Sokol, 1984) was not detected during this study. The initial study by Sokol and Woods (1983), demonstrating the presence of the 14 kDa protein, relied on the incubation of OM preparations with ⁵⁹Fe-pyochelin prior to SDS-PAGE analysis. It does however, seem unlikely that binding of ⁵⁹Fe to pyochelin or of ⁵⁹Fe-pyochelin to receptor protein would withstand SDS denaturation unless the links between molecules were covalent, nor did the authors show a profile after growth in iron replete media. The Calgary group have cloned the ferripyochelin binding protein (fbp) gene from *P. aeruginosa*. A 14 kDa protein was expressed weakly in the OM of *E. coli* (MacDougall and Sokol, 1990). Nucleotide sequencing has revealed two open reading frames, ORF1 and ORF2, each coding for an 18 kDa precursor protein. Chloramphenicol acetyl transferase (CAT) promoter fusions have been constructed to determine which promoter is active (Saravolac, 1991). An ORF1-CAT fusion resulted in CAT activity, but this was not regulated by iron. Analysis of one of the putative transcripts shows a protein with a 34 amino acid leader peptide and two potential TonB boxes (Dennis *et al.*, 1991). Interestingly, Poole *et al.*, (1990) have shown that an 80 kDa IRMP is expressed on the surface of a pyoverdinin-

deficient mutant of PAO1, when grown in the presence of enterobactin, suggesting that an enterobactin uptake system may become operative under appropriate conditions.

1.3.1.2 *Deprivation of nutrients other than iron.*

Whilst there is little information concerning the deprivation of nutrients other than iron in the body, there is evidence *in vitro* to suggest that other metal ions, including magnesium, zinc and phosphate, may be limiting (reviewed by Brown and Williams, 1985b).

Induction or repression of a variety of OMPs is a common response of bacteria in such conditions, some of which may be involved in uptake mechanisms for the nutrient required and may therefore represent an adaptive phenomenon related to host environmental conditions. A 48 kDa protein P (OprP) is expressed under phosphate limitation (Hancock *et al.*, 1982). Mutational studies have demonstrated that OprP is an important component of the high affinity, phosphate-starvation-inducible, phosphate-specific transport system of *P. aeruginosa* (Poole and Hancock, 1986). In addition, expression of the cloned OprP gene in *E. coli* is regulated by the phosphate content of the medium (Hancock *et al.*, 1990). *E. coli* produces a similar phosphate regulated protein, PhoE (36 kDa), possessing channels selective for charged molecules including phosphorylated compounds (Hancock *et al.*, 1990).

A channel-forming protein NosA has been purified from the outer membrane of *P. stutzeri* which is involved in the uptake of copper from the external medium. The expression of this OMP can be repressed by exogenous copper, although this channel does not appear to be specific for the uptake of copper (Lee *et al.*, 1989). Magnesium deficiency results in enhanced expression of protein H1 (OprH) in *P. aeruginosa* (Nicas and Hancock, 1980), with a concomitant increase in resistance to gentamicin, polymyxin and EDTA. Magnesium depletion results in a much simplified OMP profile for *P. cepacia*, compared to low media iron and nutrient broth-grown cells (Anwar *et al.*, 1983c). *P. aeruginosa* recovered directly from the CF infected lung strongly expressed OprH (Brown *et al.*, 1984), which can be induced by magnesium deprivation (Nicas and Hancock, 1980). This suggests that the lung environment is deficient in magnesium, subsequently, this may influence susceptibility of

the bacteria to aminoglycoside drugs. *K. pneumoniae* grown in continuous culture exhibited other specific proteins under potassium, carbon, sulphate and phosphate limitations, namely, 90, 48, 41, 36 kDa, respectively (Sterkenburg *et al.*, 1984).

The relative expression of the major OM porin proteins OmpF and OmpC of *E. coli* are dependent on the composition of the growth medium, *in vitro*, although the sum of the amounts appears constant (Van Alphen and Lugtenberg, 1977). Porin protein OmpC is favoured in a medium of high osmolarity and OmpF porin in low osmolarity (Van Alphen and Lugtenberg, 1977). Although bacterial porins have been shown to serve as non-specific sieves for substances with a molecular weight up to 600, their function *in vivo* is incompletely understood. The expression of OmpC as the predominant porin (smaller in pore size than OmpF) in the urinary tract, may represent an adaptation of the organism to survival in a medium of high osmolarity (Robledo *et al.*, 1990). Additionally, by expressing one instead of several porins the organism could become less recognisable to the host immune system (Robledo *et al.*, 1990).

1.3.2 Effect of sub-MICs of antibiotics on surface properties.

Dosage schedules of antimicrobial agents are often determined by use of parameters which have little bearing on drug levels found at sites of infection. Values such as the MIC and serum levels are frequently obtained using cells grown by conventional methods in the laboratory, that bear little or no resemblance to the same organisms in infection, which may affect their response to drug treatment. Consequently, antibiotics are often present in subinhibitory concentrations (sub-MICs) in infection (Mandell and Afnan, 1991).

Exposure of bacteria to sub-MICs of drugs may cause alterations in bacterial morphology (Lorian and Ernst, 1987) and proliferation, as well as, changes in the sensitivity of the organisms to serum (Atkinson and Amaral, 1982). It is apparent that at these concentrations, drugs act in a manner different from MIC, not merely causing reduced effects of the latter (Brown and Williams, 1985b). For example, filament formation is seen in many Gram-negative bacteria at sub-MICs of β -lactam antibiotics (Lorian and Atkinson, 1984). Increased serum sensitivity of *E. coli* after growth in sub-MICs of mecillinam was

suggested to be due to reduced envelope polysaccharide content (Taylor *et al.*, 1982). Sub-MICs of many antibiotics result in increased phagocytosis (Bassaris *et al.*, 1984; Lorian and Atkinson, 1984; Mandell and Afnan, 1991) and reduce the adhesive properties of many Gram-negative bacteria (Ofek *et al.*, 1979; Bassaris *et al.*, 1984). Gemmell (1987) presents *in vitro* evidence that sub-MICs of β -lactam antibiotics can expose increased numbers of specific antigenic epitopes on the bacterial cell surface, which may increase the host's clearance mechanisms of such bacteria and produce a more efficient immune response. Marked changes in immunogenic properties of bacteria have been observed previously by Leying *et al.*, (1986), however, the role of these effects in therapeutic situations is at present unknown. Although the primary targets of most antibiotics are known, there are few reports on the secondary effects on bacteria *in vivo* (Taylor *et al.*, 1982). Such secondary effects are of interest, especially in regard to sub-MICs of antibiotics, which can appear in body fluids and tissues during the course of chemotherapy (Leying *et al.*, 1986).

In an attempt to mimic *in vivo* growth conditions more closely, Kadurugamuwa *et al.*, (1985a) examined the effects of sub-MICs of cephalosporins on properties of *K. pneumoniae* grown under iron-depleted conditions. The drug concentrations used had no effect on growth rate. Enterobactin production and capsule formation were both markedly reduced and surface hydrophobicity increased following treatment. Subsequent studies demonstrated directly that loss of capsule exposed more protein antigens, including the IRMPs, on the surface, so that they become readily accessible to antibodies (Kadurugamuwa *et al.*, 1985b). Such effects may influence the susceptibility of the cells to opsonisation and phagocytosis.

Both *E. coli* recovered directly from the peritoneum of lethally-infected guinea pigs (Griffiths *et al.*, 1983) and *P. mirabilis* and *K. pneumoniae* strains harvested from infected human urines (Sciortino and Finkelstein, 1983) showed differences in expression of OMPs other than those related to iron. The former possessed additional proteins in their OM, the latter had fewer porins when compared to *in vitro* grown cells.

Exoenzymes, phospholipase C, exotoxin A, the proteases and in particular, elastase and exoenzyme S are important virulence factors for *P. aeruginosa* lung infection (Woods and

Sokol, 1986; see section 1.5). Aminoglycosides in sub-MICs, inhibit the secretion of proteases *in vitro* (Dalhoff and Doring, 1985; Geers and Baker, 1987) and ciprofloxacin, tobramycin and ceftazidime have been shown to reduce the levels of five *P. aeruginosa* exoenzymes, both *in vitro* and *in vivo* in the rat lung model for chronic local *P. aeruginosa* infection (Dalhoff and Doring, 1985; Grimwood *et al.*, 1989).

Despite the current lack of knowledge in this area, all the information accumulated thus far has established that cells grown in conventional laboratory media may not reflect microbial behaviour *in vivo*. If improved strategies for controlling and eliminating such infections are to be sought then studies of bacteria obtained directly from sites of infection or cultivated *in vitro* under conditions more closely mimicking the *in vivo* environment are essential (Brown *et al.*, 1991).

1.3.3 Effect of growth rate.

Early measurements of the doubling times of *Salmonella typhimurium* and *E. coli* in mice (Maw and Meynell, 1968; Polk and Miles, 1973) have led to the impression that multiplication of pathogens *in vivo* is slow, especially at the beginning of infection (Brown and Williams, 1985b; Ruben, 1986). This has been interpreted as indicating that nutritional conditions *in vivo* may be limiting. Undoubtedly for some pathogens multiplication is slow. More recent determinations of growth rates *in vivo* suggest, however, that slow multiplication rates and limiting growth conditions are by no means universal (Smith, 1990).

There are no well-proven, universally applicable methods for measuring doubling times of bacterial pathogens in tissues. The first attempts to measure true doubling times *in vivo* relied on a genetic marker distributing to only one of two daughter cells in each succeeding generation (Maw and Meynell, 1968; Polk and Miles, 1973). The proportion of the bacterial population carrying non-replicating markers, such as superinfecting phage or an abortively transduced phage, examined at intervals during infection, revealed the number of ensuing generations. The marker was assumed not to affect either multiplication or death of the organism. The doubling time of *S. typhimurium* was reported as being 5 to 10h in the

spleens of mice. In mouse muscle, *E. coli* failed to multiply for 4h after inoculation and doubled only twice in the next 3h (Polk and Miles, 1973). These figures contrast with doubling times of 20- 30min in laboratory media. This approach relies on the assumptions that the cells divide equally with or without the phage and that they have equal survival probability. However, it is known that sensitivity to host defences and antibiotics may vary considerably depending on the presence or absence of phage (Eudy and Burrous, 1973).

Doubling times have been calculated from the progressive loss of specific activity of bacterial populations harvested from animals at intervals after injecting radiolabelled pathogens. This method does not distinguish viable from non-viable, non-phagocytised organisms and radiolabel can leach from both, leading to an overestimation of the efficiency of the host clearance system (Morris Hooke *et al.*, 1985). For 7h after injection into the kidneys of mice, *P. mirabilis* and *E. coli* appeared to double every 2.3h and 0.9h, respectively, followed by a considerable increase to approximately 20h in the subsequent 41h period (Eudy and Burrous, 1973).

More recently, the increase in ratio of wild-type organism (which multiply *in vivo*) to those of temperature-sensitive mutants (which should not multiply *in vivo*) have been used to calculate doubling times. Tests are performed to indicate that mutants and wild-types have the same susceptibility to host defences. In the initial stages of bacterial invasion *P. aeruginosa* has been shown to achieve doubling times of 20min in the peritoneal cavity and 30min in the lung (Morris Hooke *et al.*, 1985; Sordelli *et al.*, 1985). These doubling times at the beginning of infection *in vivo* are similar to those seen *in vitro*. The authors suggest that the observed differences in growth rates may be due to varying conditions such as pH and nutrient availability. Furthermore, the media used to cultivate the initial inocula may well affect the growth rate values obtained, particularly in the first few hours after injection.

The status of the host's defence mechanisms also influences the replication rate; the doubling times in the lungs of mice rendered granulocytopenic was increased to 16min (Sordelli *et al.*, 1985) and may represent the environment of the leukaemic patient. The iron-restricted nature of the *in vivo* environment may contribute to the slower growth rate of

some bacteria. For example, certain strains of *E. coli* had doubling times of 28min when grown *in vitro* in iron-depleted media, but in the presence of transferrin or lactoferrin the doubling time was reduced to 38min (Bullen *et al.*, 1968). In experimental peritoneal infection sublethal doses of *E. coli* typically grew for a few hours and then declined. However, if the animals were injected with iron-containing compounds the same dose multiplied much more quickly and killed the animals (Bullen *et al.*, 1968). The growth rate of invading bacteria is paramount in determining the outcome of an infection, the shorter the doubling time the sooner the attainment of a bacterial population sufficient to harm the host.

The results of these animal model studies suggest that bacteria grow slowly in many chronic infections. Whilst deprivation of nutrients may contribute to the slow growth rate *in vivo* other factors including the host's immune response will also be important. The immunosuppressed leukaemic host, with a high serum transferrin saturation may, therefore, represent an *in vivo* environment in marked contrast to that observed previously in the burn wound, urinary tract infection and the CF patient.

Chemostat culture allows growth rate to be controlled with minimal changes in the physicochemical environment of the cells. Many workers have used chemostats to evaluate the effects of growth rate on envelope properties and susceptibility of cells to antibiotics, disinfectants and preservatives (Brown *et al.*, 1990). The alterations observed at different rates of growth are often dependent on the limiting nutrient. Examples include, the reduced LPS content of *P. aeruginosa* with increasing growth rate in both carbon- and magnesium-limited cultures, the level of LPS determining the degree of penetration of the cell envelope by chlorinated phenols (Gilbert and Brown, 1978). In addition, fast growing cells contained less phospholipids and more fatty acids than slow growing cells. Dodds *et al.*, (1987) have described more specific alterations in the LPS of *E. coli* grown in continuous culture at different growth rates. It was found that under both carbon and magnesium limitation much less high molecular weight LPS was produced at high dilution rates and that the small amount of O-polysaccharide present lacked amino sugars and therefore, differed chemically from that produced at low growth rates. A 39 kDa peptidoglycan-associated protein of *K. pneumoniae* expressed under carbon limitation at fast growth rates was found to be repressed when the growth rate was reduced (Lodge *et al.*, 1986). Furthermore, those

authors found that lowering the iron content of carbon-limited chemostat cultures resulted in induction of IRMPs, only at the fast growth rate. *P. aeruginosa* grown in continuous culture under magnesium limitation, at a slow growth rate, was more resistant to phagocytosis and associated cationic proteins than when growth rate was increased (Finch and Brown, 1978) and an increase in susceptibility to polymyxin and EDTA was found as the growth rate was increased (Finch and Brown, 1975). The sensitivity of *E. coli* to polymyxin B has been assessed for chemostat grown cells at specific growth rates and under various growth limiting nutrients. Magnesium- and phosphorus-limited cells demonstrated a trend of increased resistance with increasing growth rate, whereas carbon- and nitrogen-limited cells increased their sensitivity as growth rate increased (Wright and Gilbert, 1987).

Wu and Livermore (1990) compared the activities of several anti-pseudomonal β -lactams, principally ceftazidime and imipenem, against *P. aeruginosa* chemostat cultures. The killing rate by β -lactams were growth-rate-dependent with fast-growing cells being killed more rapidly than slow-growing ones. The antibiotics ceftizoxime and ceftriaxone have been shown to exhibit no activity against slowly growing cultures of *E. coli*, irrespective of the growth-limiting nutrients studied (Cozens *et al.*, 1986). The aminoglycoside antibiotics tobramycin and streptomycin are also growth-rate-dependent in their action (Muir *et al.*, 1984; Raulston and Montie, 1989) as are the newer quinolone agents (Zeiler, 1985; Zeiler and Endermann, 1986). Such effects have also been reported to influence profoundly the immunogenicity of microbes (Anwar *et al.*, 1985; Brown and Williams, 1985b) as well as susceptibility to host defences (Finch and Brown, 1978; Gilbert and Brown, 1980; Anwar *et al.*, 1983a).

Thus, it is well recognised that slowly growing bacteria possess an OM that is characteristic of their growth rate (Brown, 1977; Brown *et al.*, 1990). The composition of this membrane is markedly different in slow-growing bacteria compared to their quickly growing counterparts, emphasising the plasticity of the cell envelope.

1.3.4 Current therapy of *P. aeruginosa* infection.

In a leukaemic patient with suspected bacterial infection, antibiotics are started often before bacteria are isolated and the diagnosis confirmed. The aim is to prescribe an antibiotic regimen that offers a spectrum of activity against the most likely organisms. Selection depends on the prediction of the bacteria involved and on their susceptibility or resistance to specific antibiotics. A broad-spectrum regimen is used when the organisms involved are difficult to predict or where failure of a narrow spectrum regimen would have serious consequences as in the leukaemic patient (table 1.3.4; adapted from Bodey, 1986).

Table 1.3.4 General principles in the management of fever in neutropenic patients.

-
- 1) Temperature of 101°F (38.5°C) persisting for more than two hours unassociated with administration of pyrogenic substances indicates presence of infection until proved otherwise.
 - 2) The characteristic signs and symptoms of infection are often absent.
 - 3) Nevertheless, a careful examination that includes the oral cavity, genitalia, and anus may reveal the site of infection.
 - 4) Untreated infection will disseminate rapidly and terminate fatally.
 - 5) Delays in administration of appropriate antibiotic therapy results in suboptimal response rates.
 - 6) Consequently, antibiotic therapy must be instituted promptly with a broad-spectrum regimen.
 - 7) Most infections are caused by Gram-negative bacilli.
 - 8) “Non-pathogenic” organisms can cause serious infections and must not be ignored.
 - 9) Despite *in vitro* activity, some antibiotics are ineffective.
 - 10) Initial antibiotic selection should be influenced by the prevalence and susceptibility patterns of organisms within the hospital.
-

1.3.4.1 *Gentamicin*.

Despite the introduction of a number of new wide-spectrum antibiotics, aminoglycosides such as gentamicin remain an important therapeutic modality in the febrile neutropenic patient with a suspected bacterial infection (Tapper and Armstrong, 1974; Bodey, *et al.*, 1978; Bodey, 1984).

The accumulation of aminoglycoside antibiotics by bacterial cells has been extensively reviewed, but, the fundamental mechanisms by which these compounds are transported across the cell envelope to their ribosomal targets remains obscure (Bryan and Van den Elzen, 1976; 1977; Hancock, 1981a; 1981b; Davis, 1987; Brown and Reeves, 1992).

Several investigators have demonstrated that accumulation of gentamicin occurs in three consecutive phases (Bryan and Van den Elzen 1977; Davis, 1987). An initial ionic binding to cells is followed by two energy-dependent phases termed EDP1 and EDP2. EDP1 represents a slow rate of energised uptake; its duration and the rate of uptake are dependent on the concentration of aminoglycoside presented to the cell. The highly charged lipid insoluble aminoglycoside is subsequently transported across the cytoplasmic membrane barrier to higher-affinity ribosomal binding sites. The rapid, energy-dependent accumulation of aminoglycoside following EDP1 is referred to as EDP2 and is completed with the onset of inhibition of protein synthesis and eventual loss of cell viability.

The target site for aminoglycosides appears to be the 30S ribosomal subunit. Within this subunit, a single ribosomal protein S12 is involved in the interaction of aminoglycoside with the ribosome, but does not represent the actual binding site. The isolation of mutants, which contain alterations in ribosomal proteins, has provided additional information about the high affinity binding site for aminoglycosides. It is thought to be a three-dimensional region of the 30S subunit, which is influenced by or involves a number of different ribosomal proteins from both 30S and 50S subunits (Hancock, 1981a).

Several activities are elicited by the aminoglycosides in Gram-negative bacteria. Among these pleiotropic effects it is well established that in *P. aeruginosa* aminoglycosides interfere

with protein synthesis at the ribosomal level, the site of interaction being specific for a given antibiotic (Ahmad *et al.*, 1980; Davis *et al.*, 1986), as well as evoking damage to the outer surface (Hancock *et al.*, 1981b; Peterson *et al.*, 1985; Davis *et al.*, 1986). The mechanism of interaction of aminoglycosides with the cell envelope and its relevance in lethal action is less clear (Raulston and Montie, 1989).

When an aminoglycoside is added to a suspension of bacteria, electrostatic binding to anionic sites distributed on the cell surface occurs almost instantaneously (Bryan and Van den Elzen, 1977). This ionic binding is reversible, concentration dependent, not influenced by inhibitors of energised uptake and tends to neutralise the cell's net negative surface charge (Hancock, 1981b). Uptake of polycationic antibiotics, such as aminoglycosides, across the OM of *P. aeruginosa* has been postulated to occur via the self-promoted uptake pathway (Hancock, 1981b; Nicas and Hancock, 1983a; Hancock *et al.*, 1991). This uptake mechanism involves interaction with and displacement of Mg²⁺ ions from LPS binding sites by the polycationic aminoglycosides. Thus, the divalent cation cross-bridging of LPS is disrupted causing localised destabilisation of the OM. Alternatively, aminoglycoside uptake may occur by passing through the hydrophilic channels of porin proteins (Nikaido, 1976; Hancock, 1981a; Hancock and Bell, 1988).

During the second phase of uptake, EDP1, the aminoglycosides bind to a cytoplasmic membrane site by a process requiring active involvement from oxidised quinones and possibly also an energised membrane (Hancock, 1981a). After binding, translocation across the cytoplasmic membrane occurs. The reaction involved in translocation is relatively slow, dependent on external aminoglycoside concentrations and perhaps also on the transmembrane electrical potential gradient which is kinetically unfavourable in the absence of intracellular binding sites (Taber *et al.*, 1987).

EDP2 represents an accelerated linear rate of aminoglycoside transport across the cytoplasmic membrane by a process that uses energy, from electron transport and possibly from adenosine 5'-triphosphate (ATP) hydrolysis. The induction of EDP2 requires aminoglycoside-sensitive ribosomes engaged in protein synthesis (Taber *et al.*, 1987). The progressive binding of aminoglycoside to the high affinity ribosomal binding site results in

the acceleration of uptake, seen towards the end of EDP1. Upon saturation of the target sites (which probably include ribosomes, thus affecting protein synthesis, as well as a membrane-bound protein), it is postulated that an event occurs at the level of the membrane which contributes to but is not alone sufficient to cause lethality. The rapid influx of aminoglycoside causes progressively increasing membrane damage resulting eventually in leakage of cytoplasmic components. Further, it is proposed that the slow uptake observed in streptomycin resistant cells either represents binding to the outside of the cytoplasmic membrane or reflects lack of high affinity ribosomal binding sites but presence of other binding sites of lower affinity (Bryan and Van den Elzen, 1976; Hancock, 1981b).

The increasing frequency of emergence of gentamicin resistance in clinical isolates of *P. aeruginosa* renders treatment strategies complex (Legakis *et al.*, 1979) and poses a major clinical problem (Nilsson *et al.*, 1987). Resistance to the cationic aminoglycoside antibiotics remains an incompletely understood phenomenon. Development of resistance can be due to production of aminoglycoside inactivating enzymes (Hancock, 1981a; Bryan, 1988), to a modification of the three-dimensional region of the 30S subunit, rendering it less susceptible to binding by aminoglycosides (this type of resistance is observed less frequently; Bryan and Van den Elzen, 1976), or to impermeability to the drug (Gilleland *et al.*, 1989).

Enzymatic modification of the drug is the most common form of resistance to these antibiotics. There are three main types of aminoglycoside inactivating enzymes (Bryan, 1988; Brown and Reeves, 1992); Acetyl transferases (AAC), phospho-transferases (APH) and nucleotidyl transferases (ANT). Low levels of these inactivating enzymes need only be produced in most Gram-negative bacteria as penetration during EDP1 is very slow (Bryan and Van den Elzen, 1976). Resistant strains have been shown to exhibit the energy-independent phase and EDP1, but not EDP2, suggesting that alteration of ribosomal binding site or covalent modification of aminoglycosides during transport reduces the affinity of the drugs for the ribosome (Taber *et al.*, 1987). Evidence suggests that modifying enzymes are located in or on the cellular membrane (Taber *et al.*, 1987).

Conversely, Gilleland *et al.*, (1989) were unable to detect aminoglycoside-inactivating

enzymes in any of the three resistant strains used. It was concluded that resistance was due to an impermeability type of resistance mechanism i.e. the resistant strains grew more slowly than the parent strain, the resistance was unstable in the absence of the antibiotic and adaptation to one of the antibiotics conferred cross resistance to other antibiotics. Several authors have suggested that alterations in LPS remains the most likely basis for adaptive resistance to the aminoglycosides (Shearer and Legakis, 1985; Gilleland *et al.*, 1989). This correlates with the observation that strains which overproduce the OMP H1 (OprH) have been found to be cross-resistant to aminoglycosides, polymyxin B and EDTA (Nicas and Hancock, 1980). It is proposed that OprH replaces the divalent cation Mg^{2+} at a site on the LPS and protects this site from attack by interaction with antibiotic binding sites (Brown, 1975; Hancock, 1981a; Hancock *et al.*, 1981; Nicas and Hancock, 1980; 1983a; Bell *et al.*, 1991). In addition, Bryan *et al.*, (1984) stated that impermeability-type resistance results from conversion of smooth LPS to superficial or deeper rough LPS phenotypes. The negative charge present on LPS have implicated this polysaccharide as a possible site for interaction with the cationic aminoglycosides (Angus *et al.*, 1982; Peterson *et al.*, 1985). Therefore, EDTA, cationic aminoglycosides and polymyxin antibiotics all act at cationic binding sites on LPS, so that various conditions such as growth in Mg^{2+} deficient medium (Brown and Melling, 1969) and an increase in OM protein H1 confer resistance to all three agents.

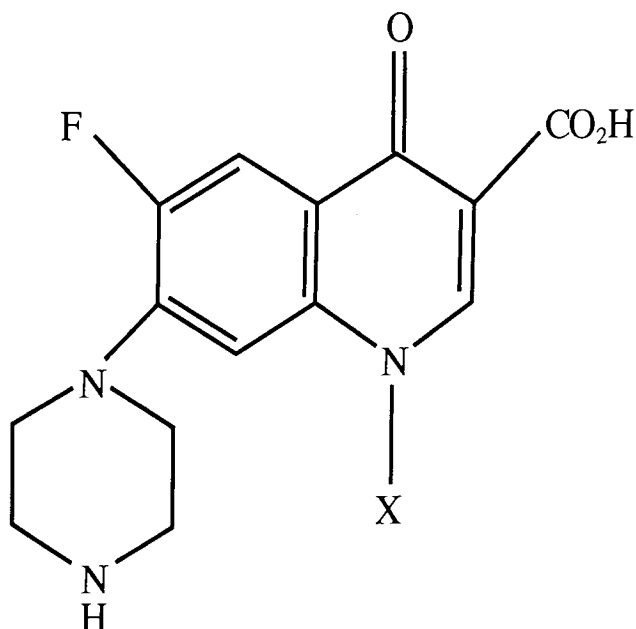
1.3.4.2 *Ciprofloxacin.*

Ciprofloxacin and norfloxacin are 4-quinolone antibacterial drugs (Fig 1.3.4.2). The fluorinated carbon at position 6 and the piperazine ring at position 7 are common both to ciprofloxacin and norfloxacin and considerably enhances the activity of these drugs in comparison with nalidixic acid (Smith, 1984a; Schentag and Domagala, 1985; Neu, 1990). These agents are of synthetic origin as opposed to those derived from fungal species, e.g. β -lactams (Smith, 1984a).

Clinical efficacy of an antibacterial drug is derived from a combination of several factors including antibacterial activity, mechanism of action and achievable serum and tissue concentrations. Ciprofloxacin achieves a maximum serum concentration of about $2-3mg.L^{-1}$

with the oral dosage schedule of 500mg twice daily (Drusano, 1989). Moreover, its tissue penetration is good and drug concentrations achieved in most tissues are of the same order of those in serum (Campoli-Richards *et al.*, 1989). Norfloxacin achieves a maximum serum concentration of about 1.5-2mg.L⁻¹ with an oral dosage schedule of 400mg twice daily (Lode *et al.*, 1990). Peak serum levels are above 1mg.L⁻¹ and therefore, exceed the MIC for many common pathogens (Hooper and Wolfson, 1985). These agents are highly active bacterial agents, effective against a broad spectrum of Gram-positive and Gram-negative bacteria (King *et al.*, 1984; Lode, 1990; Hooper and Wolfson, 1991b) including *P. aeruginosa*, for which it has a very low MIC (Morris and Brown, 1988). Ciprofloxacin is more active than norfloxacin against most bacterial species, the differential being, in general, about eight-fold (Reeves *et al.*, 1984b).

Fig 1.3.4.2



where X= CHCH₂CH₂ for ciprofloxacin.

= CH₂CH₃ for norfloxacin.

The molecular events leading to death of bacteria exposed to the 4-quinolones are not known. However, differences have been observed amongst these drugs in the conditions

required for their lethal action. Three independent mechanisms of action A, B and C have been found to be responsible for the bactericidal activity of the 4-quinolones against *Escherichia coli* KL16 (Lewin and Smith, 1986).

Mechanism A was the first bactericidal mechanism to be identified. It is exhibited by all the 4-quinolones and is the sole bactericidal mechanism of the older drugs, such as nalidixic acid (Smith, 1984b). Protein and RNA synthesis and dividing bacteria are prerequisites. Therefore, drugs such as the protein synthesis inhibitor chloramphenicol or the RNA synthesis inhibitor rifampicin, completely abolish the bactericidal activity of nalidixic acid against *E. coli* (Deitz *et al.*, 1966; Smith, 1984b).

Studying the effect of rifampicin or chloramphenicol on the bactericidal activities of the 4-quinolones led to the identification of a second bactericidal mechanism, termed B. A bacteriostatic concentration of either chloramphenicol or rifampicin was found to reduce, but not to abolish the bactericidal activity of ofloxacin or ciprofloxacin against *E. coli* KL16 (Ratcliffe and Smith, 1984). Mechanism B, exhibited by ciprofloxacin does not require protein or RNA synthesis for its activity and is active against non-dividing bacteria (Zeiler and Grohe, 1984; Chalkley and Koornhof, 1985; Smith and Lewin, 1988).

Mechanism C, exhibited by norfloxacin is also active against non-dividing bacteria but, unlike mechanism B, protein and RNA synthesis are required for its bactericidal activity (Ratcliffe and Smith, 1985). Hence it can be abolished by addition either of chloramphenicol or rifampicin (Ratcliffe and Smith, 1985). It has been suggested that possession of several mechanisms of action may explain why the newer generation of 4-quinolones are more potent than the older drugs, which exert only a single mechanism of action (Smith, 1984b) and also why mutational resistance is less frequent with the modern drugs (Smith, 1986a). The number of bactericidal mechanisms which are exhibited by a 4-quinolone would seem to be important in determining its clinical usefulness (Lewin and Smith, 1988).

The primary target for the antibacterial activity of the quinolones is believed to involve inhibition of bacterial DNA gyrase (topoisomerase II; Gellert, 1981; Kayser, 1985; Hooper

et al., 1987). This essential enzyme introduces negative superhelical twists into DNA (Gellert *et al.*, 1976) and is responsible for the accommodation of the long circular chromosome within the limited space provided by the cell envelope (Furet and Pechere, 1990).

DNA gyrase is composed of two dimeric subunits and contains two 105 kDa A subunits encoded by the *gyrA* gene and two 90 kDa B subunits encoded by the *gyrB* gene (Higgins *et al.*, 1978). It is believed that the A subunits first promote strand separation by introducing nicks into each strand of the double-stranded chromosomal DNA (Gellert *et al.*, 1977), the DNA is then supercoiled by the B subunits. A molecule of ATP is used for each twist that is produced (Morrison and Cozzarelli, 1979). Finally, the resultant negative supercoils are locked into each domain by the A subunits, which are thought to seal the nicks that they first introduced (Gellert *et al.*, 1977). These authors proposed that the 4-quinolones somehow prevented the A subunits of DNA gyrase from finally sealing the staggered nicks they first introduced into chromosomal DNA, which accords with the suggestion made previously (Crumplin and Smith 1976). However, it is well known that mutations affecting the B subunit of DNA gyrase, changes bacterial sensitivity to the 4-quinolone antibacterials (Inoue *et al.*, 1978; Yamagishi *et al.*, 1986). Hence both subunits may be targets for the 4-quinolones (Hooper *et al.*, 1987; Hooper and Wolfson, 1991a). The bactericidal effect of the 4-quinolones has been explained by the fact that the inhibited DNA gyrase does not seal the nicks it has made in the chromosome. These interruptions could then induce the synthesis of exonucleases which could enlarge the gaps until they become permanent double strand excisions (Fenwick and Curtiss, 1973; Hill and Fangman, 1973; Crumplin and Smith, 1975; Wolfson and Hooper, 1985). Yet, exactly how quinolones interfere with DNA gyrase function remains controversial. Do quinolones exert their effect by binding to DNA gyrase? Such a hypothesis is supported by the observation of the enzymatic inhibition of purified DNA gyrase by quinolones (Barrett *et al.*, 1990) and bacterial resistance associated with DNA gyrase mutations (Gellert *et al.*, 1977; Sato *et al.*, 1986). However, if quinolones bind directly to DNA gyrase the exact binding site remains to be determined. Other reports have suggested that quinolones bind to DNA and not directly to DNA gyrase (Shen and Pernet, 1985) or to the tertiary DNA gyrase-DNA complex (Shen *et al.*, 1989). At clinically achievable concentrations, quinolones would not

seem to inhibit human topoisomerase II (Hussy *et al.*, 1986). This is due probably to structural and functional differences between the two enzymes and would seem to provide an explanation of why the 4-quinolones are toxic to bacteria, but not to humans (Smith and Lewin, 1988).

Bacterial resistance to an antimicrobial agent may be mediated either via a plasmid-encoded factor or a chromosomal mutation (Lewin, 1992). Plasmids are molecules of extra-chromosomal DNA that can replicate independently of the chromosome and may be freely transferred between bacteria, including those of different species (Lewin *et al.*, 1990). Plasmids can mediate antibacterial resistance by one of three mechanisms:

- 1) Inactivation of the drug e.g. β -lactams/ β -lactamases.
- 2) Production of an additional target resistant to the antimicrobial agent e.g. trimethoprim/dihydrofolate reductase.
- 3) Active efflux of the drug from the cell e.g. tetracycline/RNA ribosome complex.

It is usual for plasmids to carry a number of genes conferring resistance to many antibiotics. As a result of this plasmids are usually the main carriers of resistance in many clinical bacteria (Amyes, 1989). Plasmid-mediated resistance to the modern 4-quinolones has not yet been identified (Smith, 1986b; Piddock and Wise, 1989), leaving chromosomal mutation to account for most emergent 4-quinolone resistance (Burman, 1977). It has been shown that chromosomally-mediated resistance to the 4-quinolones occurs by one of two mechanisms. Either an alteration in the target enzyme DNA gyrase or a mutation causing an alteration of the OM resulting in a reduction in the permeability of the cell for the drug (Hooper *et al.*, 1987; Robillard and Scarpa, 1988; Smith and Lewin, 1988). This latter mechanism may prevent cellular penetration of other antimicrobial agents besides the quinolones and thus confer cross-resistance. Resistance may be reversible after 4-quinolone therapy is discontinued. This may be due either to loss of factors coding for resistance within a pathogenic strain or the disappearance of a resistance clone otherwise disadvantaged in the presence of a more dominant strain. There is evidence for both phenomena, but none to suggest which is the more dominant (Ogle *et al.*, 1988).

Although clinical resistance to all the new quinolones has been encountered, the overall frequency has been low and has not necessarily been associated with clinical failure (Neu, 1988). Spontaneous high-level resistance (>8-fold higher MIC) to the new fluoroquinolones is selected in a single step at a frequency of 10^{-7} to 10^{-9} (Chin and Neu, 1984; Sanders *et al.*, 1984; Cullmann *et al.*, 1985; Daikos *et al.*, 1988). This may well reflect a requirement for sequential multiple mutations prior to expression of clinically relevant resistance. Development of resistance or a reduced susceptibility to ciprofloxacin therefore, is uncommon in most bacteria with the possible exception of *P. aeruginosa* (Eron *et al.*, 1985; Chamberland *et al.*, 1989; Wolfson and Hooper, 1989). These resistant strains were usually encountered among patients whose ability to fight infection was compromised by defects of their immune system or in patients suffering from chronic infection (Campoli-Richards *et al.*, 1989). This higher mutation rate, coupled with a sometimes low therapeutic ratio with *P. aeruginosa*, suggests that single-step mutation to resistance might result in treatment failure (Wolfson and Hooper, 1989). It is important to continue to monitor patients closely to determine how often resistant bacteria emerge.

1.4 COMPOSITION OF GRAM-NEGATIVE BACTERIAL ENVELOPES.

Bacteria have a remarkable ability to alter their metabolism in response to changing environmental conditions. The prime structure through which this adaptation is mediated is the bacterial cell envelope. For pathogenic bacteria, cell envelope structures are involved in promoting adhesion to and colonisation of host tissues, in the acquisition of essential nutrients and in conferring resistance both to host defence mechanisms and to antibiotics used to treat such infections (Brown and Williams, 1985a; 1985b; Dalhoff, 1985; Nikaido and Vaara, 1985; Smith, 1977; 1990). The envelope phenotype expressed by a bacterium is a product of its growth environment and this ability to adapt to its surroundings confers major survival advantages on the cell (Brown and Williams, 1985b; Williams, 1988). The envelope can be divided into the inner cytoplasmic membrane (CM), the peptidoglycan layer (PG), the periplasm, the outer membrane (OM), the extracellular polysaccharide layer and surface appendages. A schematic representation of the cell envelope is shown (Fig 1.4; adapted from Sleytr *et al.*, 1988).

Fig 1.4

Schematic representation of the OM of Gram-negative bacteria.



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From Sleytr *et al.*, (1988).

The CM is a lipid bilayer composed of phospholipids and containing embedded proteins. Both its inner and outer faces are hydrophobic due to partly exposed fatty acid moieties of its constituent phospholipids (Costerton *et al.*, 1979). This membrane contains components of the electron transport chain and oxidative phosphorylation proteins involved in the active transport of solutes and excretion of waste products, in addition to the synthetic apparatus necessary for the production of exterior layers (Hammond *et al.*, 1984).

The PG encloses the CM, conferring shape and rigidity on the cell, enabling it to withstand osmotic pressure. PG consists of a backbone of N-acetyl muramic acid and N-acetyl glucosamine molecules cross-linked by short peptide chains to form a rigid polymer (Hammond *et al.*, 1984).

The region in the Gram-negative envelope situated between the CM and OM is known as the periplasmic space. It is composed of hydrated PG, more highly cross-linked than the inner PG layer (Hobot, 1984). Here oligosaccharides are involved in the regulation of osmolarity and 3 classes of low molecular weight proteins; those with a catabolic function which convert nutrients into a form to enable transportation, those which bind nutrients and those which act as enzymes involved in degradation of harmful substances including antibiotics (Hammond *et al.*, 1984).

Surrounding the periplasm are the two OM components most relevant to this study, LPS and OMPs and these are therefore, discussed in more detail (section 1.4.1 and 1.4.2). Since the focus of the work is on *P. aeruginosa* the following sections concentrate on this organism, although details of *E. coli* and *K. pneumoniae* will be included where appropriate.

1.4.1 Outer membrane proteins.

A number of proteins have been identified and characterised in the OM of *P. aeruginosa* (Hancock and Nikaido, 1978; Mizuno and Kageyama, 1978). The nomenclature assigned by Hancock *et al.*, (1990) has been generally adopted. It is well established that many of the proteins are expressed only under specific growth conditions, frequently in response to

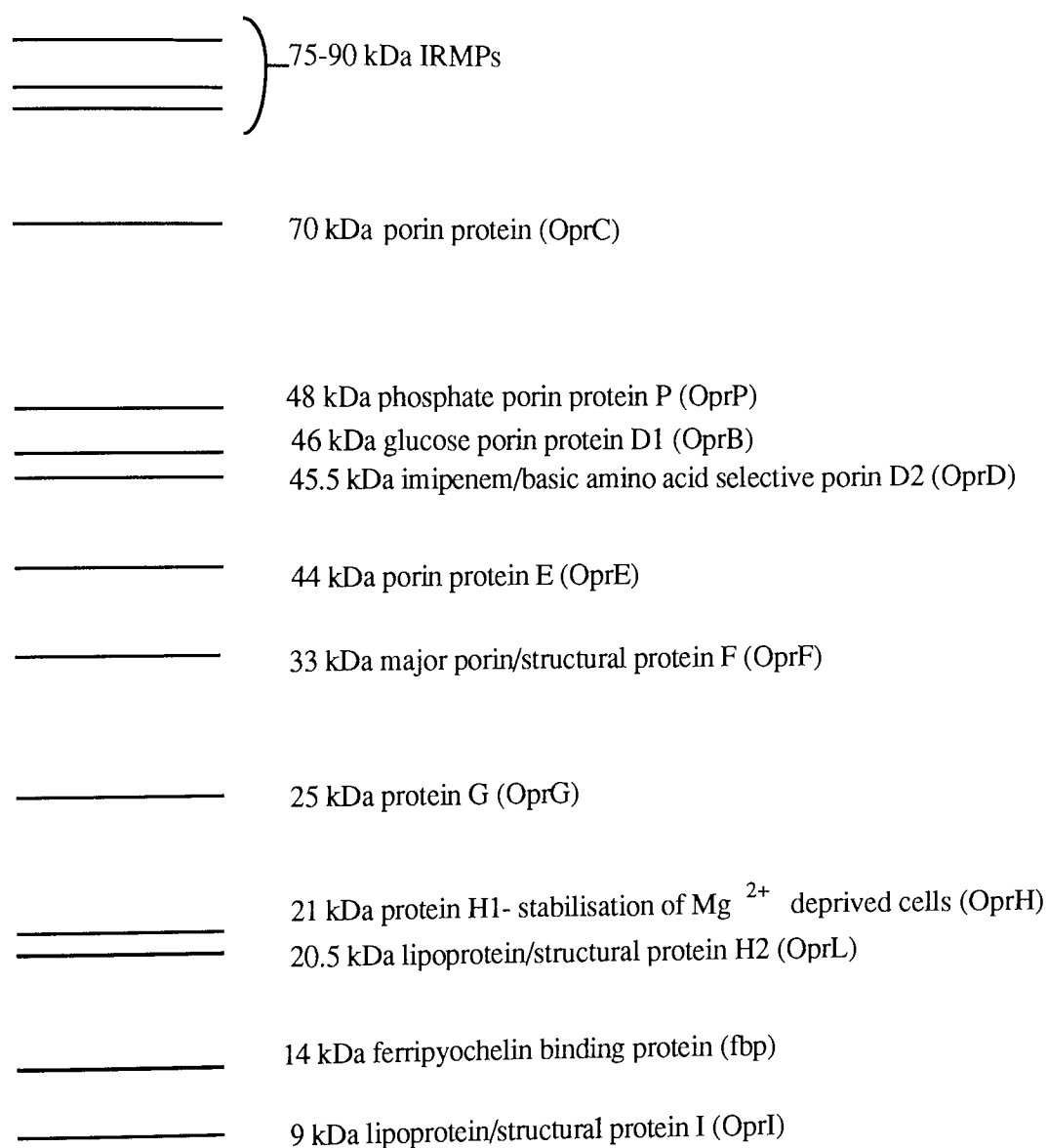
the availability of particular nutrients. Representations of the OM therefore resemble only a 'snap-shot' taken under particular conditions of growth rate and nutrient availability.

The proteins have been characterised mainly according to their migration pattern following SDS-PAGE (Fig 1.4.1). Among these proteins OprB, D, F, G and H are all heat modifiable, since their electrophoretic mobility differs depending on the temperature of denaturation (Hancock and Carey, 1979). OprB, D, G and H show a decrease in mobility on SDS-PAGE (i.e. an increase in their apparent molecular weights) on heating between 70-100°C in SDS. This arises as a result of structural rearrangements within the protein molecule under denaturing conditions, such that the apparent molecular weight is increased, rather than due to degradation (Hancock and Carey, 1979). OprF is not included in this classification and requires prolonged boiling in SDS and 2-mercaptoethanol for complete denaturation. The extensive β -sheet structure of this protein (Mizuno and Kageyama, 1979b) may be involved in the modification.

Proteins C (OprC) and E (OprE) of *P. aeruginosa* have been purified from OMs. Liposome swelling techniques have been used to demonstrate that they are general porins (Yoshihara and Nakae, 1989). The authors suggest a small exclusion limit; only solutes having a molecular weight of less than 250 being able to penetrate easily. Recently, it has been claimed that OprC plays a predominant role in the influx of negatively-charged cephalosporin antibiotics across the *P. aeruginosa* membrane (Satake *et al.*, 1990). However, this conclusion is based on the comparison of liposome swelling rates obtained with OprC and an apparently inactivated OprF. In addition, demonstration of rapid penetration of cephalosporins with molecular weights as high as 644, contradicts the earlier conclusions from the same group, that even disaccharides with a molecular weight of 342 cannot diffuse across the *P. aeruginosa* OM (Yoneyama and Nakae, 1986; Yoshihara *et al.*, 1988; Yoshihara and Nakae, 1989).

Proteins D1 (OprB) and D2 (OprD) have very similar molecular weights (Hancock and Carey, 1979) and were not identified as separate polypeptides in earlier studies (Mizuno and Kageyama, 1979b). OprB is coregulated with the glucose uptake system of *P. aeruginosa* and with a periplasmic glucose-binding protein (Hancock and Carey, 1980). It has been

Fig 1.4.1



Pattern of OMPs of *P. aeruginosa* following separation by SDS-PAGE on 12% acrylamide gels after denaturation at 100°C in SDS and mercaptoethanol. The molecular weight and function of the proteins are shown, where known (adapted from Hancock *et al.*, 1990).

shown to function as a porin (Hancock and Carey, 1980) and liposome swelling studies have suggested a preference of the channel for glucose and xylose over other sugars of similar size (Trias *et al.*, 1988). OprB has been compared to the lam B protein of *E. coli* due to the proposed involvement of both in periplasmic binding, protein-mediated carbohydrate uptake systems, and due to their similar mode of induction (Hancock and Carey, 1980).

P. aeruginosa mutants resistant to the broad spectrum carbapenem β -lactam, imipenem, have been isolated both from clinical sources and in the laboratory. They lack an OMP of molecular weight 45 to 46 kDa (Quinn *et al.*, 1986; Buscher *et al.*, 1987; Gotoh and Nishino, 1990). This OMP has recently been identified by Trias and Nikaido (1990b) as porin OprD. While the purified protein is capable of allowing size-dependent uptake of monosaccharides in liposome-swelling assays (Yoshihara and Nakae, 1989), it appears to demonstrate selectivity for imipenem over other β -lactams based on the lack of cross resistance of OprD mutants to other β -lactams (Quinn *et al.*, 1986) and on whole cell OM permeability and enzyme-encapsulated liposome data (Trias *et al.*, 1989) comparing OprD mutants and wild type. Thus, OprD has the characteristics of a substrate-selective porin i.e. one with a specific binding site for imipenem. It seems likely that these are natural analogues e.g. basic amino acids (Trias and Nikaido, 1990a).

The function of OprF as a *P. aeruginosa* porin is controversial. Liposome exclusion (Hancock *et al.*, 1979) and liposome swelling data (Yoshimura *et al.*, 1983; Trias *et al.*, 1988; Nikaido *et al.*, 1991), have shown it to function as a porin. These studies suggest that the exclusion limit is large (molecular weight 3,000), but that they have very few porin molecules containing channels that allow the passage of large molecules. This explanation is consistent both with the relatively large size of some β -lactams (molecular weight > 500) that are known to enter and kill *P. aeruginosa* and with model membrane studies in which OprF preparations were shown to contain large channels at a low frequency and small channels at a high frequency (Woodruff *et al.*, 1986; Woodruff and Hancock, 1988). In addition, it has been proposed that the intrinsic resistance of *P. aeruginosa* results from the fact that more than 99% of OprF molecules are nonfunctional (Angus *et al.*, 1982; Nicas and Hancock, 1983b; Woodruff *et al.*, 1986). In contrast to these findings, other

investigators have suggested that the *P. aeruginosa* OM has a low exclusion limit resulting in impermeability to disaccharides and that OprF has no function as a porin (Yoneyama and Nakae, 1986; Yoshihara *et al.*, 1988; Gotoh *et al.*, 1989; Yoshihara and Nakae, 1989). However, Nikaido *et al.*, (1991) have proposed that in the latter studies, OprF was inadvertently inactivated during purification. The authors suggest that the 'conflict' between the large pore size and the low overall permeability is only apparent and that consideration of the pore shape, may explain both the large exclusion limit and the low permeability of the *P. aeruginosa* porin channels. Although, OprF is non-covalently linked to the underlying peptidoglycan layer (Mizuno and Kageyama, 1979b), this protein must span the OM, since Lambert and Booth (1982), using ¹²⁵I-labelled peroxidase, demonstrated the surface location of this protein. This has been further confirmed by indirect immunofluorescence techniques with monoclonal antibodies to OprF (Mutharia and Hancock, 1983).

OprF has an important function in cell shape determination and OM stability (Gotoh *et al.*, 1989). A recent finding that the amino acid sequence of *P. aeruginosa* OprF shows some homology to that of *E. coli* OmpA (35 kDa), suggests that they may be functionally related (Woodruff and Hancock, 1988; 1989). In addition, OprF shares the following characteristics with the OmpA protein; they have similar molecular weights, strong peptidoglycan and LPS association and substantial stable β -sheet structure and they are similarly heat modifiable on SDS-PAGE (Hancock and Carey, 1979; Mizuno and Kageyama, 1979b; Yoshimura *et al.*, 1983; Woodruff and Hancock, 1988). In addition, Nikaido *et al.*, (1991) suggest that OmpA does produce pores and can facilitate transmembrane diffusion of sugars as large as disaccharides. However, the properties of the channel exhibits similarities to those of OprF in that the permeability was still very low. It seems likely that *P. aeruginosa* OprF and *E. coli* OmpA are representatives of a class of porins different from the classical porins represented by OmpF (37.2 kDa), OmpC (36 kDa) and PhoE (38 kDa) porins of *E. coli*. The former apparently exist as monomers, produce large channels, but of low permeability, in contrast to the latter, which exist as stable trimers producing narrow channels of high permeability (Nikaido *et al.*, 1991).

The appearance of OprG in the OM of *P. aeruginosa* is strongly dependent on growth conditions. For example, iron-sufficient conditions, growth into the stationary phase,

higher growth temperatures, Mg²⁺ deficiency, certain LPS alterations, the presence of specific carbon sources and adaptive resistance to antibiotics, have all been shown to alter levels of OprG in the OM (Hancock and Carey, 1979; 1980; Nicas and Hancock, 1980; Ohkawa *et al.*, 1980; Kropinski *et al.*, 1987; Gilleland *et al.*, 1989). These considerations make assignment of a function to OprG difficult at present, however, roles in fluoroquinolone uptake (Chamberland *et al.*, 1989) or in low-affinity iron-uptake (see chapter 4) have been suggested.

Proteins H1 (OprH) and H2 (OprL) share similar molecular weights and were not resolved in some of the earlier studies using SDS-PAGE, so these proteins were designated protein H (Mizuno and Kageyama, 1979b). OprH is induced in the OM of *P. aeruginosa* when grown in media deficient in Mg²⁺, Ca²⁺, Mn²⁺ and Sr²⁺ to become a major OMP (Nicas and Hancock, 1980; 1983a). This envelope phenotype has been associated with increased resistance to polymyxin, EDTA and aminoglycoside antibiotics (Nicas and Hancock, 1980; 1983a). OprH has been proposed to replace Mg²⁺ at LPS divalent cation, cross-bridging sites in the OM and block self-promoted uptake of these polycationic antibiotics (Nicas and Hancock, 1980; 1983a; Hancock, 1990).

P. aeruginosa OprL is strongly associated with peptidoglycan and contains covalently bound fatty acid chains and thus belongs to the class of OMPs called peptidoglycan-associated lipoproteins (Mizuno, 1981). When whole cells of *P. aeruginosa* PAO1 were reacted with ¹²⁵I-labelled peroxidase, OprL was shown not to be surface exposed (Lambert and Booth, 1982). In addition, monoclonal antibodies to OprL did not bind to intact smooth *P. aeruginosa* cells, but did bind to rough LPS-deficient mutants. This was thought to be due to unmasking of OM components (Mutharia and Hancock, 1983).

Protein I (OprI) in the OM of *P. aeruginosa* is also a lipoprotein and is analogous to the Braun lipoprotein of *E. coli* (7.2 kDa; Mizuno and Kageyama, 1979a). It is rich in α helical structure (Mizuno and Kageyama, 1979b) and exists in both free and covalently bound forms in the OM (Mizuno and Kageyama, 1979a).

OprL, the peptidoglycan-associated lipoprotein and OprI, the Braun lipoprotein equivalent,

have no ascribed functions to date, since no mutants lacking these proteins have been isolated (Woodruff and Hancock, 1989). However, OmpA together with the Braun lipoprotein have been demonstrated to have a structural role in the stability and maintenance of shape of *E. coli* (Sonntag *et al.*, 1978). The expression of IRMPs and OprP are discussed in section 1.3.1.1 and 1.3.1.2, respectively.

1.4.2 Lipopolysaccharide.

Lipopolysaccharide (LPS), a major component of the OM of Gram-negative bacteria, is important in the structure and function of this membrane (Kropinski *et al.*, 1985). Structurally, this amphipathic molecule comprises three genetically, biochemically and antigenically distinct domains, the lipid A, the core oligosaccharide and the O-antigen polysaccharide, which are covalently bound to each other.

The lipid A component from different species of Gram-negative bacteria share a general structure based on a phosphorylated β 1-6 glucosamine disaccharide, to which fatty acids are attached by ester and amide bonds (Hammond *et al.*, 1984). LPS molecules have 6 or 7 fatty acids linked to the glucosamine disaccharide backbone (Nikaido and Vaara, 1985). Lipid A is unique among bacterial fatty acids in containing hydroxy fatty acids (Hammond *et al.*, 1984). The absence from lipid A of 3-hydroxytetradecanoic acid (3OH-C_{14:0}) and the presence of 2-hydroxydodecanoic acid (2OH-C_{12:0}), 3-hydroxydodecanoic acid (3OH-C_{12:0}) are characteristic of *P. aeruginosa* (Wilkinson and Galbraith, 1975). The lipid A component appears to assume a highly ordered conformation on the cell surface, with the fatty acid chains being tightly packed in a dense hexagonal lattice and the diglucosamine backbone forms domains in a parallel arrangement in the membrane. The high state of order of lipid A may be an important factor, in the structural role and permeation barrier functions of LPS in the OM (Labischinski *et al.*, 1985).

The core is a complex oligosaccharide consisting of an inner and an outer region. The inner core is relatively conserved, comprised mainly of L-glycero-D-manno-heptose, KDO, phosphate and ethanolamine. Additional characteristic components include D-glucose, L-rhamnose, D-galactosamine and L-alanine, although proportions of the respective

components differ, due to environmental conditions and to a degree between strains (Wilkinson, 1983). The unique sugar, KDO links the core polysaccharide to lipid A via an acid labile bond (Wilkinson and Galbraith, 1975).

The outer core region of *P. aeruginosa* differs significantly from that of enteric species. It contains glucose, rhamnose, heptose, galactosamine and possibly alanine, KDO and high concentrations of phosphate (Wilkinson, 1983; Kropinski *et al.*, 1985). The heptose constituent of *P. aeruginosa* contains equal amounts of L-glycero-D-manno-heptose and D-glycero-D-manno-heptose (Hammond *et al.*, 1984). The core LPS region of *P. aeruginosa* is more highly phosphorylated than that of other Gram-negative bacteria (Wilkinson and Galbraith, 1975) and strain PAO1 has been estimated to contain 10 phosphate groups (Kropinski *et al.*, 1985). Phosphate groups in the KDO-lipid A region of LPS have a high affinity for divalent cations (Mg^{2+} and Ca^{2+}) forming cross-bridges which are important in maintaining the structural integrity of the OM (Wilkinson, 1983). *P. aeruginosa* is unusually sensitive to EDTA and this feature is probably a result of chelation of the cations, so destabilising the membrane (Wilkinson and Galbraith, 1975).

The O-antigen and the core oligosaccharide form the hydrophilic portion. The O-side chain consists of repeating polysaccharide sugar units, which are extremely variable (Jann *et al.*, 1975; Hitchcock and Brown, 1983) and form the basis of the immunological typing of serotypes (Bergan, 1975). The O-specific side chains of *P. aeruginosa* are rich in rhamnose glucose and the amino sugars, hexosamine, fucosamine and quivosamine (Wilkinson, 1983). The smooth to rough morphology mutation coincides with the disappearance of O-antigen leaving a rough LPS consisting only of core oligosaccharides bound to lipid A (Hammond *et al.*, 1984). The proportion of LPS molecules with attached side chains and the number of subunits in the side chain are dependent, in part, on media composition and growth temperature (Kropinski *et al.*, 1985; 1987; McGroarty and Rivera, 1990).

Few studies have considered the phenotypic variability of *P. aeruginosa* LPS. Gilbert and Brown (1978) reported reductions in the KDO content of *P. aeruginosa* LPS with increasing growth rate and in addition, magnesium-limited cultures contained more KDO than glucose-limited cells from chemostat cultures. In addition Dodds *et al.*, (1987) found under both

carbon and magnesium limitation in continuous culture *E. coli* produced less high molecular weight LPS than at low dilution rates. Day and Marceau-Day (1982) investigated the effects of growth rate and magnesium depletion on the KDO and heptose content of *P. aeruginosa*. Their results indicated that these components varied independently of one another and with growth rate and nutrient conditions.

Bacterial lipopolysaccharide, including those from *P. aeruginosa* possess numerous and varied biological properties. The exposed O-saccharide side chain is primarily responsible for the strong immunogenicity of LPS, whereas the endotoxic response is associated with the lipid A anchored in the outer face of the OM (Wilkinson, 1983). In addition, they contribute to the distinct properties of the bacterial OM (Chester and Meadow, 1975; Goldman and Leive, 1980; Palva and Makela, 1980) and provide receptors for some bacteriophages and bacteriocins (Wilkinson, 1983). Furthermore, they are directly involved in the uptake of cationic antibiotics and in the bactericidal action of EDTA on the organism.

1.5 HOST RESPONSE TO INFECTION.

Host defence against bacterial infection involves the complex interaction between non-specific and specific mechanisms. Non-specific defences encompass a wide range of factors aimed at preventing entry of microorganisms into the host, including the barrier function of the skin, the acidity of gastric juices and the protective actions of secretions, bathing surfaces such as the conjunctiva of the eyes and the lungs. Microorganisms which succeed in gaining entry to body sites are presented with an environment hostile to their proliferation. This is achieved, in part, by withholding essential nutrients, in particular reducing the level of iron available to bacteria (see section 1.2.1) and by production of a spectrum of serum components which serve as mediators of inflammatory reactions and fever. Specific immune defences rely on recognition of invading microbial antigens and the subsequent coordinated response of antibodies, phagocytic cells, lymphocytes and complement. These defences interfere with bacterial growth, promote the uptake and killing of bacteria by phagocytes and inhibit the toxic properties of bacterial exoproducts (Young and Armstrong, 1972).

When considering host defence mechanisms against a bacterial species, virulence factors must also be considered. Virulence factors can be divided into two general groups, cell surface components and extracellular products. Surface components of *P. aeruginosa* important in pathogenesis include LPS, proteins, pili and alginate (Vasil, 1986). Extracellular factors which contribute to the virulence of *P. aeruginosa* in lung infections include proteases, exotoxin A, hemolysins such as phospholipase C and exoenzyme S (Young, 1980; Woods and Sokol, 1986). Pseudomonal elastase, a metalloprotease with a broad substrate specificity, cleaves a number of biologically important host molecules such as elastin, collagen, immunoglobulins, some complement components and human transferrin (but not lactoferrin), releasing iron to the bacteria (Gambello and Iglewski, 1991). Pseudomonal proteases (elastase and alkaline protease) inhibit the function of phagocytes, natural killer and T lymphocytes, by cleaving the CD4 molecule on T-helper cells, inhibiting IL-1 and IL-2 activity, cleaving immunoglobulins and inactivating complement components. The proteases of *P. aeruginosa* and toxins such as exotoxin A and exoenzyme S, may be important in establishing initial colonisation by preventing the host from mounting a local immune response and by inhibiting non-specific defence mechanisms (Wick *et al.*, 1990). Baker *et al.*, (1991) have recently shown exoenzyme S to function as an adhesin. Furthermore, exoenzyme S and antibodies to it inhibit the attachment of *P. aeruginosa* to buccal cells which the authors suggest is in support of a role for this protein in adherence.

1.5.1 Humoral Immunity.

One arm of the specific immune response involves activation of B-lymphocytes. On challenge with antigens, which bind to particular receptors on the lymphocyte membrane, a complex sequence of events is initiated involving cellular differentiation and extensive protein, RNA and DNA synthesis in the bone marrow.

The antibodies formed include representatives from the 3 main immunoglobulin classes, IgG, IgA and IgM, with IgG being the most abundant. Binding of IgG to the surface of bacteria renders them more hydrophobic favouring phagocytic interaction (Stendhal, 1983). IgG alone promotes phagocytosis of bacteria by neutrophils and macrophages without the

participation of complement, but the opsonic support of phagocytosis is enhanced by complement activation. IgM antibodies are large molecules of molecular weight 900 kDa compared to 150 kDa for IgG and as such are confined to the vascular system. They are formed early in the immune response and are very powerful with respect to avidity and complement activating capacity. IgA circulates in the plasma as a monomer like IgG, but is also secreted onto mucosal surfaces as a dimer covalently bonded to a secretory component, having a molecular weight of 385 kDa. The secretory component stabilises the polymer and renders it less susceptible to proteolytic attack (McNabb and Tomasi, 1981). The major function of secretory IgA is not as an opsonising antibody but *in vivo* it appears to interfere with attachment to the mucosal membrane (Stendhal, 1983). The mechanism of its anti-adhesive effect is not well understood. Secretory IgA also inhibits antigen uptake by mucosa and neutralises toxic exoproducts of bacteria (McNabb and Tomasi, 1981).

The main antimicrobial actions of antibodies include promoting phagocytosis by acting as opsonins, preventing attachment of microorganisms to surfaces by binding to bacterial appendages involved in adhesion, reducing motility by inhibition of flagella antigens and possibly restricting uptake of essential nutrients by binding to certain OM receptors. In addition, antibodies directed against microbial toxins may neutralise the effects of such compounds. Finally, by combining with microbes, antibodies activate complement, so inducing an inflammatory response ultimately resulting in cell lysis (Mims, 1987).

1.5.2 Complement.

The complement system comprises at least 12 different proteins capable of causing lysis of invading microorganisms (Taylor, 1983). Proteins of the classical pathway have been designated C1 through C9 and the sequence can be initiated when the first component C1 binds to antigen-bound IgG or IgM antibodies. In addition to lysis of bacteria, some components (C3a) degranulate mast cells, producing local inflammation which focuses some of the body's defence mechanisms on the site of infection by attracting neutrophils; some act as chemotaxins (C3a and C5a) and some act as opsonins (C3b), which enhance the ingestion of invading bacteria by phagocytes. This latter function is one of the primary roles of the complement system. Complement may also be bactericidal when associated

with specific antibody on surfaces of certain bacterial species. The terminal proteins of the complement system, C6, C7, C8 and C9, the 'membrane-attack complex' are involved in this biological activity, destroying the membrane and causing lysis of the cell. Since a single molecule of activated C1 generates thousands of molecules of the later components, the whole series is an amplification process and is sometimes termed the complement cascade (Mims, 1987).

An alternative pathway which does not require antigen/antibody immune complexes may be activated by compounds such as bacterial endotoxin and polysaccharide, in conjunction with a number of endogenous plasma proteins, factors B, D and P (Properdin). The result is activation of the complement by the alternative pathway.

1.5.3 A coordinated response to infection.

Infection and inflammation generally result in a reduction in the plasma concentration of iron. This decrease is thought to be attributable primarily to increased storage of iron in the liver and within cells of the reticuloendothelial system (Lee, 1983) and to a lesser extent to decreased absorption of iron across the gut epithelium (Flanagan, 1989). Increased excretion of iron via the urinary tract has only a minor role in reducing the plasma concentration of iron (Kluger and Bullen, 1987).

The increased liver concentrations of iron are thought to be due to a large pool of iron in red blood cells and a smaller pool of iron bound to transferrin. Red blood cells have an average lifespan of about 120 days and senescent or damaged erythrocytes are phagocytosed by the reticuloendothelial cells. During the next several days iron from haemoglobin is released into the general circulation where it can be either used to produce new red blood cells or stored as ferritin. While the amount of iron bound to transferrin is relatively small compared to that in haemoglobin, the turnover rate of the plasma-iron pool is relatively rapid (Kluger and Bullen, 1987).

Evidence suggests that the hypoferraemia of infection or inflammation is the result of decreased release of iron from the reticuloendothelial system, rather than the increased

uptake from the plasma transferrin-iron pool (Kluger and Bullen, 1987). Lee (1983) has suggested two mechanisms for this increased storage of iron in the liver, the first is the release of lactoferrin from neutrophils and the second is the increased synthesis of ferritin. Both appear to participate in the hypoferraemic response.

Lactoferrin is a glycoprotein which at low pH has a greater affinity for iron than transferrin. Van Snick *et al.*, (1974) proposed that the hypoferraemia of infection and inflammation was the result of release of lactoferrin from neutrophils, the subsequent transfer of iron from transferrin to lactoferrin and the selective uptake of the lactoferrin-iron complex by the reticuloendothelial system. This theory is supported by the fact that interleukin-1 does result in neutrophil release of lactoferrin (Kluger and Bullen, 1987). However, the amount of lactoferrin-iron taken up by the reticuloendothelial system from the general circulation, where pH is generally about 7.4, is probably too small to account for the large fall in plasma iron concentrations. It is therefore suggested that, in response to the synthesis and release of interleukin-1, lactoferrin is released from neutrophils, complexes with iron and remains at or near its site of release. This complex is then picked up by circulating or fixed macrophages, resulting in localised hypoferraemia. Systemic hypoferraemia resulting in increased storage of iron in the liver may be brought about during general infections where interleukin-1 is released by Kupffer cells in the liver (Lee, 1983).

The second theory to explain the hypoferraemia of infection and inflammation is that synthesis of ferritin, an iron-storage protein, is increased in the liver (Konijn and Hershko, 1977). The authors showed that in rats injected with turpentine, hepatic ferritin synthesis increased and that this increase preceded the decline in plasma iron concentration, suggesting that the ferritin had bound free iron and this led to the hypoferraemia of infection and inflammation. Letendre and Holbein (1983; 1984) have shown that in mice infected with *Neisseria meningitidis* the hypoferraemic response was associated with a preferential incorporation of haem-derived iron into the intracellular hepatic ferritin pool which limits the supply of the metal to the extracellular pool.

The elevation of serum iron either artificially or otherwise generally results in increased severity of infection (see section 1.2.3). Although it is assumed that the hypoferraemia of

infection is an adaptive host defence response, no experiments have been performed to show that prevention of the reduction of iron at the actual time of infection has any effect on mortality or morbidity due to that infection. However, the overwhelming data supporting a beneficial role for the hypoferraemia of infection would suggest that prevention or an inability to reduce serum iron would adversely affect the host.

P. aeruginosa strains are generally effectively opsonised with C3b by the classical pathway and to a lesser extent by the alternative pathway (Peterson, 1980). However, to circumvent some of the many facets of the host immune defences outlined above, *P. aeruginosa* has developed certain strategies to evade the immune system. Somatic and extracellular products of *P. aeruginosa* interfere at all stages in the phagocytic process (Peterson, 1980) and mucoid strains resist the action of phagocytes even in the presence of specific antisera (Baltimore and Mitchell, 1980). Additionally, *P. aeruginosa* elastase has a destructive effect on immunoglobulins and complement. Of the latter components, only C4 and C7 are resistant to degradation by the enzyme (Schultz and Miller, 1974). Alkaline protease and elastase from *P. aeruginosa* are thus capable of depressing the inflammatory response, inhibiting chemotaxis of neutrophils and lowering phagocytic activity (Kharazmi *et al.*, 1984a; 1984b; Pedersen and Kharazmi, 1987). Most strains of *P. aeruginosa* are resistant to the bactericidal effects of serum (Taylor, 1983). However, phenotypically induced alterations in surface components of the bacterial cell may also alter the sensitivity of the bacterium to serum and complement-mediated killing (Finch and Brown, 1978; Anwar *et al.*, 1983a). For example, lack of an O-antigen side-chain has been associated with increased sensitivity of *P. aeruginosa* to opsonisation and phagocytosis (Engels *et al.*, 1985).

1.6 Aims of the study.

Restricting the availability of iron to pathogens plays an important role in mammalian host defence strategies, a tactic termed “nutritional immunity” (Bullen, 1981; Weinberg, 1978; 1984; Finkelstein *et al.*, 1983). There has been much emphasis on the necessity of iron for bacterial growth and the need of the host to sequester iron and make it unavailable. Indeed, the influence of iron metabolism, both of the invading bacterial pathogen and in the host, is

widespread and often appears to be crucial in deciding the outcome of an infection. During clinical disease, host iron metabolism may alter and infection of a compromised patient is likely to differ significantly from that in a healthy host. There is evidence that some of the serum proteins which bind iron compounds can be reduced significantly in certain clinical conditions. For example, the reduction in the level of transferrin in leukaemia (Hunter *et al.*, 1984a).

The aim of this study was to investigate aspects of the host environment of the leukaemic patient. This was achieved by designing a protocol for the selection of patients. The iron status of these leukaemic hosts was determined prior to and throughout an infection episode in conjunction with gaining additional evidence regarding the recognition of OMPs by the host's immune system during this clinical infection situation.

The project was designed to generate additional information by observing the effect of sub-MICs of the anticancer drug mitomycin C on various strains of *P. aeruginosa*. Preliminary studies indicated that OprG expression was influenced by iron and by the presence of ciprofloxacin in the growth media. Therefore, further investigations were undertaken to examine the influence of available iron and growth rate/phase on the sensitivity of *P. aeruginosa* to gentamicin and ciprofloxacin both in chemostat and batch culture.

2. MATERIALS

2.1 BACTERIAL STRAINS.

Table 2.1.1 *P. aeruginosa* strains used in this study.

STRAIN	SOURCE
PAO1 (ATCC 15692)	Aston collection
NCTC 6750	Aston collection
PAM7	Aston collection
AK1012	A.M.Kropinski
PAJ	Otitis media clinical isolate
PA 889	Ciprofloxacin resistant clinical isolate (CF patient)

Table 2.1.2 Clinical isolates from leukaemic patients used in this study.

PATIENT	STRAIN
PAVI	<i>P. aeruginosa</i>
PAJK	<i>P. aeruginosa</i>
PAGF	<i>P. aeruginosa</i>
ECSM	<i>E. coli</i>
ECVI	<i>E. coli</i>
ECHD	<i>E. coli</i>
ECFA	<i>E. coli</i>
ECHH1	<i>E. coli</i>
ECHH2	<i>E. coli</i>
KPSW	<i>K. pneumoniae</i>
KPWS	<i>K. pneumoniae</i>

All strains were maintained on nutrient agar slopes at 4°C and subcultured at approximately 3 monthly intervals. In addition glycerolised cultures were kept in liquid nitrogen.

2.2 CLINICAL MATERIAL.

Serum samples from septicaemic leukaemic patients colonised with Gram-negative bacteria were taken throughout the infection episode by Dr. N. Patton (Haematology unit, Queen Elizabeth Hospital, Birmingham). The blood was allowed to clot for 2h at 37°C and then centrifuged at 2000 x g for 10min. The supernatant serum was collected and stored at -20°C. The bacterial clinical isolates were supplied by the Microbiology Department, Queen Elizabeth Hospital, Birmingham.

2.3 CHEMICALS.

All chemicals and reagents not specified in the text were supplied by BDH Chemicals Ltd., (Poole, Dorset), Sigma Chemical Company (Poole, Dorset) and Fisons (Loughborough, Leics.) and were of Analar grade or equivalent. For *K. pneumoniae* studies, glucose, ammonium chloride and the sodium phosphate salts were Aristar grade (BDH).

The antimicrobial compounds used in this study were Ciprofloxacin (Bayer UK Ltd., Newbury, Berkshire), Gentamicin, Norfloxacin and the cytotoxic antibiotic Mitomycin C (Martindale Pharmaceuticals Ltd., Romford, Essex).

2.4 PREPARATION OF GLASSWARE.

All glassware was fully immersed in 0.01% ethylenediaminetetraacetate (EDTA) and allowed to stand overnight at room temperature. It was then rinsed five times in distilled water, followed by 5 times in double distilled water before drying at 60°C. All glassware was sterilised at 121°C for 20min.

2.5 CHEMICALLY DEFINED MEDIA (CDM).

The composition of the chemically defined growth media for *P. aeruginosa* (formulated by Noy, 1982), *E. coli* (Klemperer *et al.*, 1979) and *K. pneumoniae*, (Williams *et al.*, 1984), are shown in tables 2.5.1, 2.5.2 and 2.5.3, respectively.

The concentration of each essential nutrient is sufficient to allow exponential growth to a theoretical optical density (OD) of 10, in practice however, oxygen becomes growth rate-limiting before any of the medium constituents. Iron restriction was achieved by growing the organism in CDM to which iron had been omitted (CDM-Fe); iron-replete bacteria were grown in CDM-Fe supplemented with 10 μ M FeSO₄ (CDM+Fe). The constituents were dissolved in the appropriate volume of double distilled water and sterilised by autoclaving at 121°C for 20min. Glucose and iron were autoclaved separately for *P. aeruginosa* and *E. coli* CDM and aseptically added to the other sterile constituents.

2.6 COMPLEX MEDIA.

Nutrient agar, nutrient broth and tryptone soya broth (TSB) were obtained from Oxoid (London) and *Pseudomonas* isolation agar (PIA) from Difco (West Molesey, Surrey).

Table 2.5.1 Composition of CDM for *P. aeruginosa*.

CONSTITUENT	FINAL CONCENTRATION (mM)
KCl	0.62
NaCl	0.5
K ₂ HPO ₄ ·3H ₂ O	3.2
(NH ₄) ₂ SO ₄	40.0
MgSO ₄ ·7H ₂ O	0.4
MOPS ¹	50.0
Glucose	40.0
FeSO ₄ ·7H ₂ O ²	0.01

¹MOPS- (4-morpholino-propane sulphonic acid), adjusted to pH 7.4 with 10M NaOH.

²FeSO₄·7H₂O- (10mM) solution was acidified with 1ml concentrated H₂SO₄.L⁻¹ to prevent precipitation when autoclaving.

Stock solutions of glucose and iron were autoclaved separately and combined in sterile glassware for each experiment.

Table 2.5.2 Composition of CDM for *E. coli*.

CONSTITUENT	FINAL CONCENTRATION (mM)
$(\text{NH}_4)_2\text{SO}_4$	6.0
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.5
KCl	13.4
KH_2PO_4	28.0
$\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$	72.0
MOPS ¹	50.0
Glucose	27.0
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ²	0.005

¹ MOPS-(4-morpholino-propane sulphonic acid), adjusted to pH 7.4 with 10M NaOH.

² $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ - (10mM) solution was acidified with 1ml concentrated $\text{H}_2\text{SO}_4 \cdot \text{L}^{-1}$ to prevent precipitation when autoclaving.

Stock solutions of glucose and iron were autoclaved separately and combined in sterile glassware for each experiment.

Table 2.5.3 Composition of CDM for *K. pneumoniae*.

	CONSTITUENT	FINAL CONCENTRATION
1	Glucose	35.0mM
	NH ₄ Cl	25.0mM
	MgSO ₄ .7H ₂ O	0.4mM
	KCl	1.5mM
	NaCl	45.2mM
2	Sodium phosphate buffer pH 7.4	66.67mM
3	FeSO ₄ .7H ₂ O	20.0μM
4	CaCl ₂ .6H ₂ O	0.5μM
	H ₃ BO ₃	0.5μM
	CoCl ₂ .6H ₂ O	0.05μM
	CuSO ₄ .5H ₂ O	0.05μM
	ZnSO ₄ .7H ₂ O	0.05μM
	MnSO ₄ .4H ₂ O	0.05μM
	(NH ₄) ₆ Mn ₇ O ₂₄ .4H ₂ O	0.005μM

Stock solutions containing combinations of the constituent salts were made (table 2.5.3, 1-4). These were autoclaved separately and combined in sterile glassware for each experiment.

2.7 EQUIPMENT.

Automatic pipettes: Gilson pipetman, P-20, P-100, P-200, P-1000, and P-5000 (Anachem Ltd., Luton, Beds.).

Balances: Sartorius 1702 balance (Satorius Instruments Ltd., Belmont, Surrey), Oertling HC22 (Oertling, Orpington, Kent).

Centrifuges: MSE high speed 18, MSE superspeed 50 (Measuring and Scientific Equipment Ltd., Crawley, Sussex), Beckman J2-21, Beckman L8-60M Ultracentrifuge (Beckman Instruments Ltd., High Wycombe, Bucks.) and Eppendorf 5412 bench centrifuge (Baird and Tatlock Ltd., Atherstone, Leics.).

ELISA plates: Immulon 2 ELISA plates, flat-bottomed (Dynatech, Billingham, Sussex).

Freeze dryer: Edwards Modylo freeze dryer (Edwards High Vacuum Ltd., Crawley, Sussex).

French Press: Amicon Corp., High Wycombe, Bucks.

Gas Liquid Chromatography: Chromatography series 204 with PMB8222 dual pen recorder (Philips/Pye-Unicam, Cambridge).

Gel electrophoresis equipment: Mini-Protean system (Bio-Rad Laboratories Ltd., Watford, Herts.), or made in house (Aston Services). Additionally, a large slab gel apparatus (in house Aston Services).

Gel Drier: Model 224 (Bio-Rad, Watford, Herts.).

Immunoblotting apparatus: Trans Blot Cell (Bio-Rad Laboratories Ltd., Watford Herts.).

Incubators: Mickle Reciprocating water bath (Cam Lab Ltd., Cambridge), Gallenkamp orbital shaking incubator (Gallenkamp, London).

Laser Densitometer: Ultrascan Laser Densitometer, LKB 2202 run by an Apple II Europlus computer with gelscan software (LKB, Croydon, Surrey).

Membrane Filters: Gelman Acrodisc 0.22 μ m (Gelman Sciences, Brackmills, Northampton).

Microscopes: Wild model M20, binocular, phase contrast microscope (Micro Instruments Ltd., Oxford).

Peristaltic pump: Model 501s, Watson Marlow, Falmouth, Cornwall.

pH meter: PTI-15 digital pH meter (Fisons Scientific Apparatus, Loughborough, Leics).

Photography equipment: Nikon camera FG (Nippon Kogaku KK, Tokyo, Japan), with Kodak Technical Pan film 2415.

Power packs: Bio-Rad Model 500/200 (electrophoresis), Bio-Rad Model 250/2.5 (immunoblotting; Bio-Rad Laboratories Ltd., Watford, Herts.).

Rotary evaporator: Buchi Rotorvapor-R (Fisons Scientific Apparatus, Loughborough, Leics.).

Sonicator: MSE Soniprep 150 (Measuring and Scientific Equipment Ltd., Crawley, Sussex).

Spectrophotometers: LKB ultrospec 4050, Cecil CE292 and Unicam SP8000 scanning UV spectrophotometer (Pye Unicam Instruments Ltd., Cambridge).

Cuvettes for spectrophotometer measurements: plastic (Gallenkamp, Loughborough, Leics.), quartz (Hellma, Westcliffe on sea, Essex).

Syringes: Hamilton precision syringes 25 μ l, 50 μ l (Hamilton Bonaduz AG, Switzerland).

Whirlimixer: Fisons Scientific Apparatus (Loughborough, Leics.).

3. EXPERIMENTAL METHODS

3.1 MEASUREMENT OF BACTERIAL CELL GROWTH.

Changes in cell concentration during bacterial growth were followed using spectrophotometric measurement. At low cell concentrations the light scattered by a bacterial cell suspension is directly proportional to the cell concentration in the suspension, as expressed by the Beer-Lambert Law:

$$OD = \frac{\text{Log } I_0}{I}$$

where I_0 = intensity of incident light and

I = intensity of the emergent light provided that the light path is constant.

This relationship is obeyed between an optical density (OD) of about 0.03 and 0.3 (Kenward, 1975). Measurements of absorbance were made at a wavelength of 470nm to minimise absorption by media constituents and bacterial metabolic products such as pyocyanin. Therefore, an OD_{470nm} was used as it has been found to be the wavelength giving maximum absorbance of the culture with minimal interference from the medium. An OD of 1.0 at 470nm indicates a concentration of approximately 1×10^9 cfu.ml⁻¹ (Anwar, 1981).

3.2 GROWTH CONDITIONS.

3.2.1 Batch Culture.

Batch culture was carried out in Erlenmeyer flasks containing not more than 25% of their volume of appropriate media. Cells were grown in pre-warmed CDM (see section 2.5) at 37°C, agitated at 180rpm on a shaking orbital. Growth of bacteria was measured by determining changes in OD of the culture with time. Samples for estimation of OD were

removed aseptically at appropriate intervals, dilutions being made in fresh growth medium when necessary. Undiluted samples were returned to the flask to prevent undue reduction in volume, diluted samples were discarded.

3.2.2 Continuous Culture.

3.2.2.1 Theory.

In batch culture bacteria are subjected to a constantly changing environment. As the cell density increases, so nutrient availability decreases and secondary metabolites accumulate. The rate and extent of growth of the culture, in the absence of toxic metabolites, will therefore depend upon the physicochemical conditions and the concentration of the least available nutrient. This becomes the rate-limiting nutrient for growth and the cells then enter stationary phase. Consequently it is a closed system which is difficult to define.

The chemostat provides a method whereby bacteria can be grown at a constant and controllable growth rate in a closely-defined nutritional environment (Fig 3.2.2.1). It comprises an open system where spent media, biomass and secondary metabolites are removed at a rate equal to the input of fresh growth media (Herbert *et al.*, 1956). If the working volume of the culture (V) is kept constant, the residence time (T_R) of any given cell in the culture is a function of the dilution rate (D):

$$T_R = \frac{1}{D}$$

And:

$$D = \frac{F}{V}$$

where F is the rate of flow of fresh media into the culture vessel as determined by the pump setting and the bore size of the tubing.

The substrate concentration is kept constant by the flow of fresh media, any increase in growth rate results in a fall in the substrate concentration, thereby decreasing growth rate

and vice versa. A steady state population is reached where growth rate is controlled by the rate of supply of an essential nutrient and hence the specific growth rate (μ ; increase in cell mass) equals the dilution rate (D; loss of cell mass).

But
$$\mu = \frac{\ln 2}{t_d}$$

where t_d is the doubling time.

hence
$$D = \mu = \frac{0.693}{t_d} \quad \text{or} \quad t_d = \frac{0.693}{D}$$

Within limits microbial growth therefore takes place under steady-state conditions. Nutrient concentration, pH, oxygen, osmolarity, secondary metabolites, all of which change during batch culture can be maintained at steady-state levels in continuous culture (Herbert *et al.*, 1956). The effects of growth-rate *per se* can therefore be distinguished from other influences.

3.2.2.2 Method.

The design of the chemostat was based on that of Gilbert and Stuart (1977; Fig 3.2.2.2). Jacketed glass chemostats (approximately 50ml) at 37°C were used in these experiments. The chemostat culture was aerated by passing sterile air through a humidifier and into the culture vessel through a glass sinter at the bottom at a rate of 100ml.min⁻¹. Air was sterilised by filtration through a cellulose acetate membrane filter (0.22µm, 47mm diameter, type GS, Millipore, UK). The working volume was accurately determined by pumping medium into the chemostat via sterile soft silicone tubing, until excess medium passed via the overflow to a sterile collecting vessel. The airflow was initiated and the vessel contents were poured through the overflow into a measuring cylinder. The two chemostats used in this study were found to have working volumes of 51ml and 50ml, with *P. aeruginosa* in CDM at the cell densities used.

Fig 3.2.2.1

Schematic representation of chemostat design

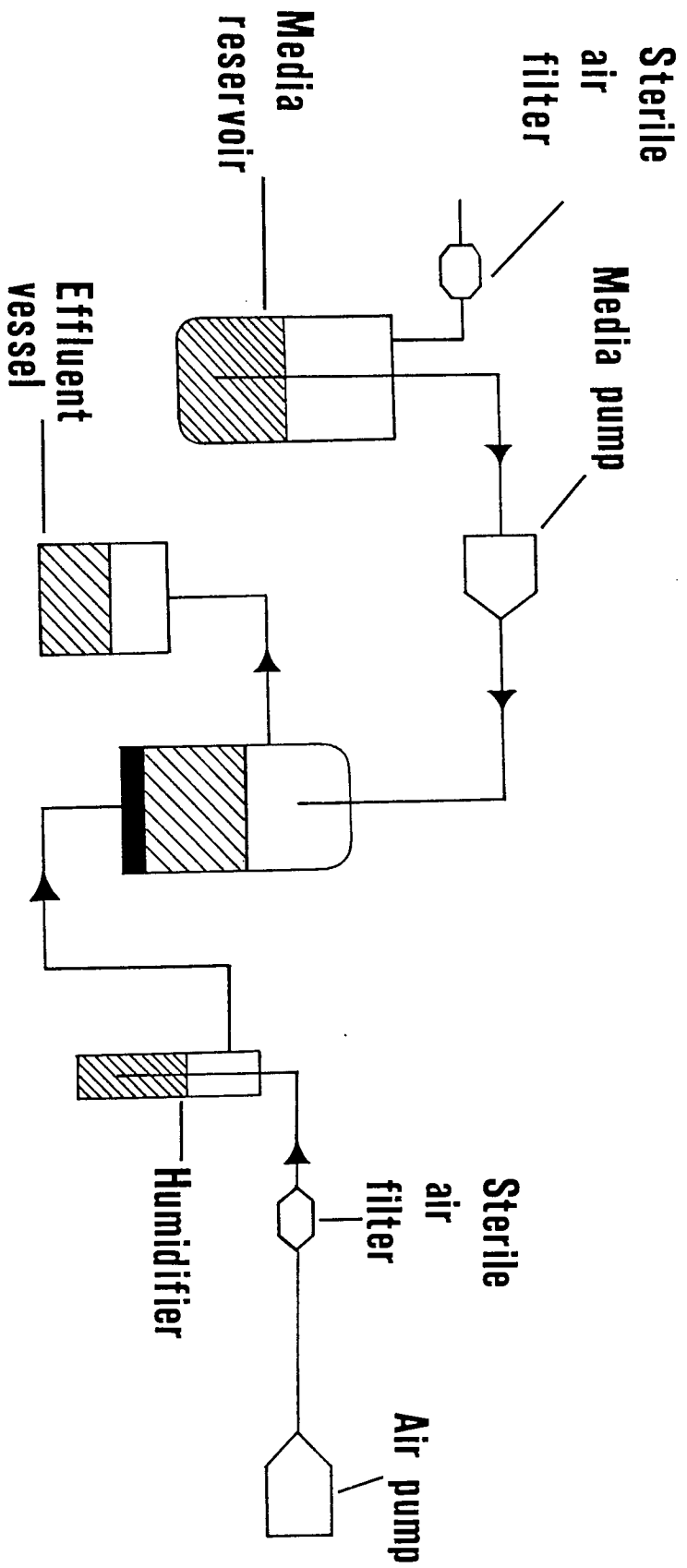
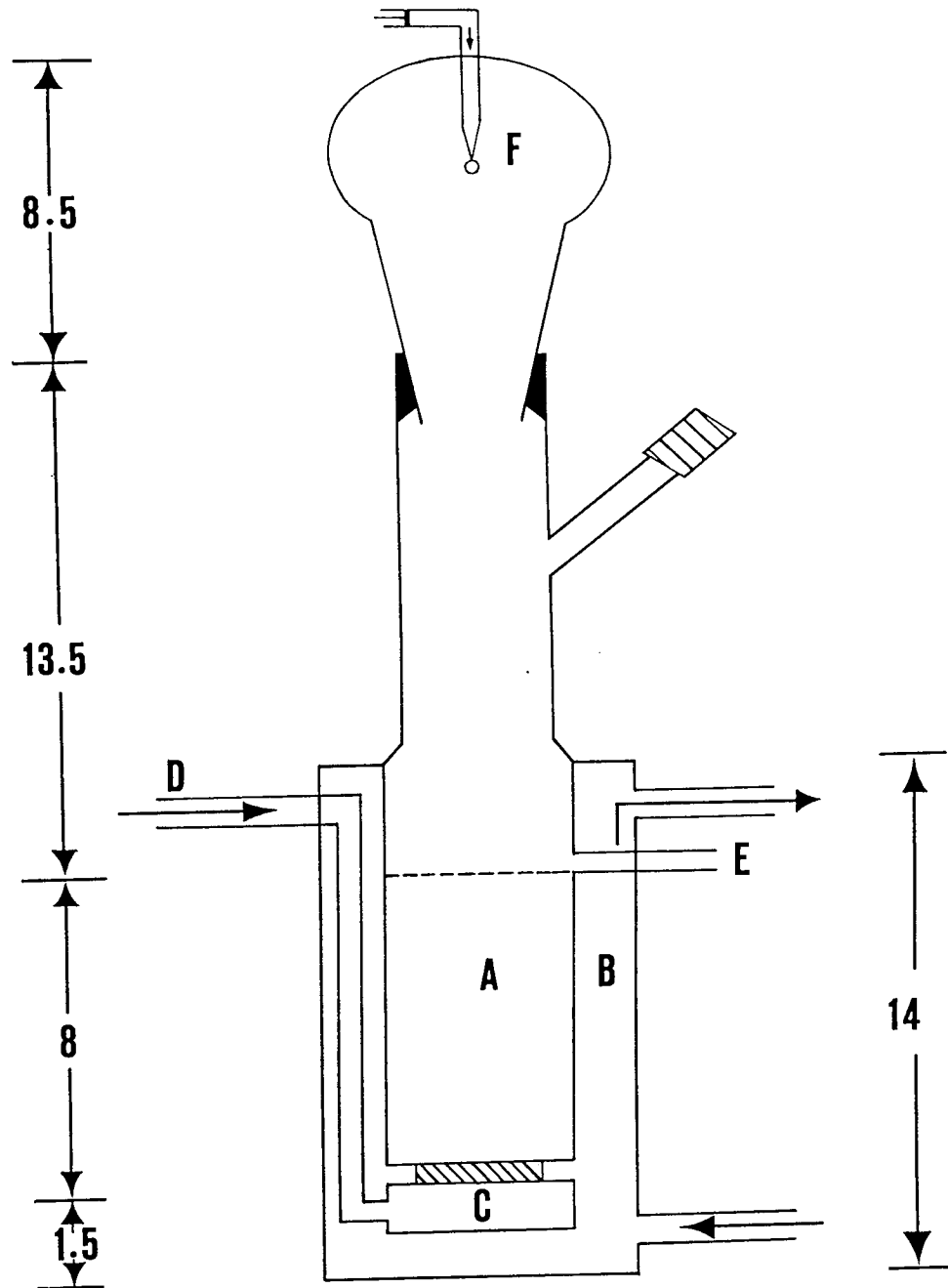


Fig 3.2.2.2

Diagram of the chemostat for use in continuous culture experiments



- A: Main culture vessel.
- B: Water jacket.
- C: Glass Sinter.
- D: Sterile air inlet.
- E: Overflow.
- F: Medium inlet tube.

Dimensions in centimetres

Since a knowledge of the dilution rate and hence of the flow rate are required for growth rate control in the chemostat, the delivery rates of the peristaltic pump were determined. The medium input tubes from the chemostat were attached to the pump with their distal ends being placed in graduated measuring cylinders. The pump was switched on at different settings (0-10) and the flow rate calculated over a period of time. These were plotted against the pump setting for 4 different size bore tubing (Fig 3.2.2.3, 3.2.2.4, 3.2.2.5 and 3.2.2.6).

Before assembly, the chemostat glassware was soaked in detergent solution (Lipsol), followed by several hot rinses and then treated as described in section 2.4. The chemostat was then filled with sterile CDM from the media reservoir using a peristaltic pump. It was then allowed to stand for 24h, with temperature control and air flow on, before being plated out as a sterility check. A mid-log culture (500 μ l) was aseptically added to the chemostat vessel and allowed to grow overnight, as a batch culture. The pump was switched on the following day, initially at a slow flow rate which was increased progressively until the desired rate was reached. Once steady state was achieved (in all experiments at least 5 volume changes were allowed), sampling was accomplished where possible by direct removal from the culture vessel. When large volumes were required samples were collected on ice from the overflow.

At various steady-state dilution rates ($D= 0.11 \text{ h}^{-1}$, $D= 0.28 \text{ h}^{-1}$, $D= 0.41 \text{ h}^{-1}$, $D= 0.44 \text{ h}^{-1}$), optical density ($OD_{470\text{nm}}$) and viable counts were determined. Figure 3.2.2.7 illustrates the changes in steady-state optical density of a chemostat culture of *P. aeruginosa* PAO1 grown under iron-limiting conditions in CDM-Fe at various dilution rates and figure 3.2.2.8 shows changes in steady-state viable count in the same system. As the dilution rate (D) is increased the optical density and viable count were found to be dependent directly upon it. It is difficult to compare OD with viable counts because of the light scattering properties of the larger cells obtained at higher growth-rates (Lodge, 1987). In addition, at higher growth rates the proportion of viable cells is likely to increase, but this will not necessarily affect optical density readings which reflect total cell count. This increase was not evident in this study.

Fig 3.2.2.3

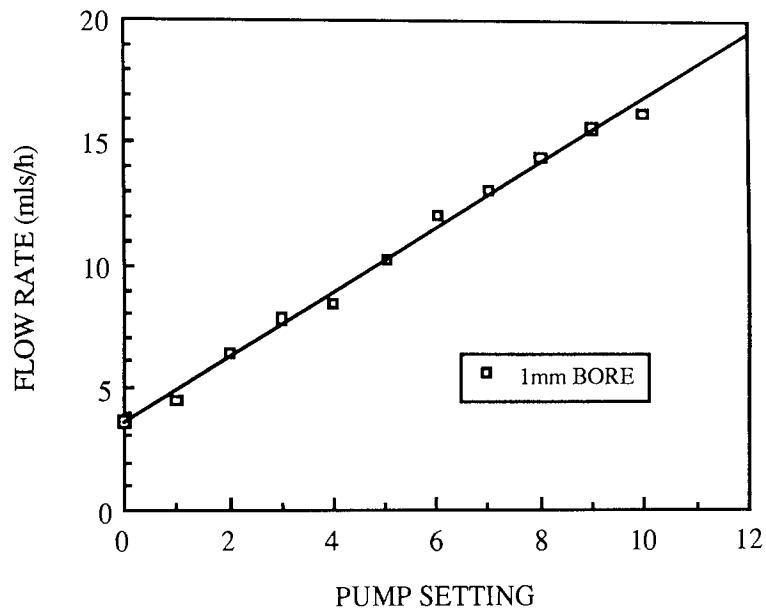
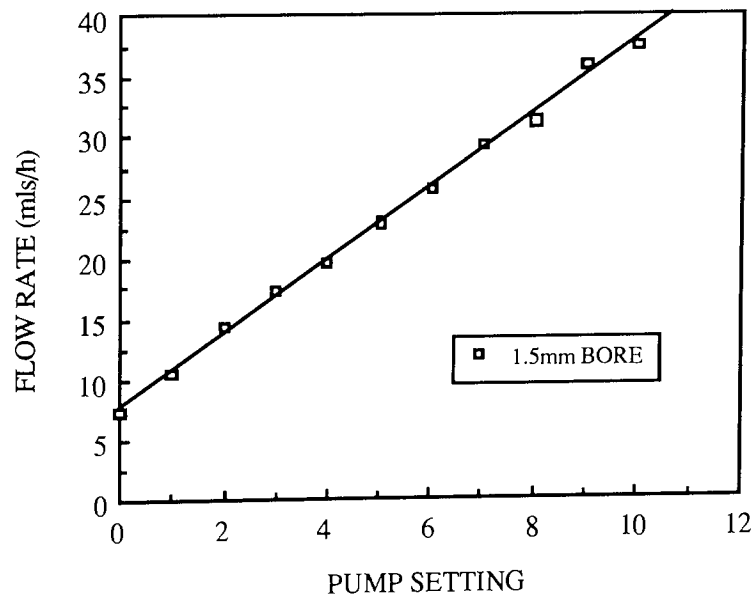


Fig 3.2.2.4



Flow rates obtained with the peristaltic pump with different tube bores.

Fig 3.2.2.5

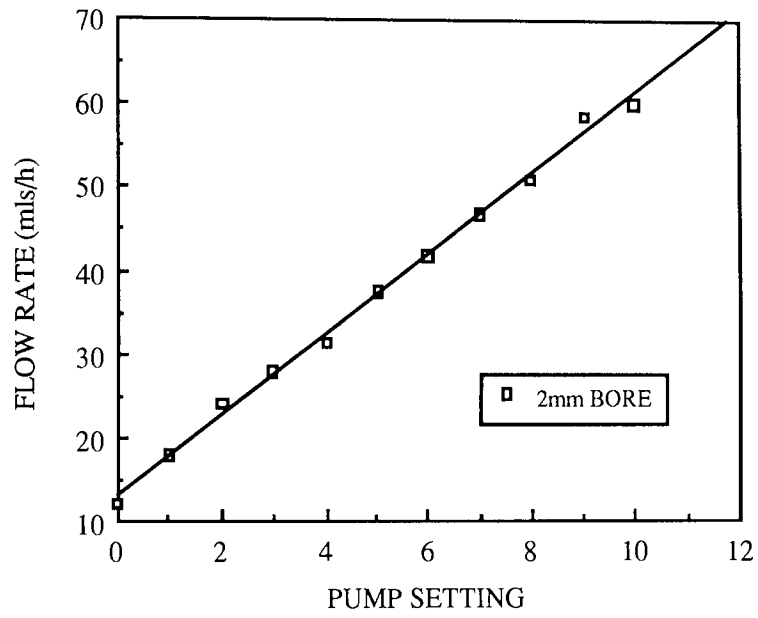
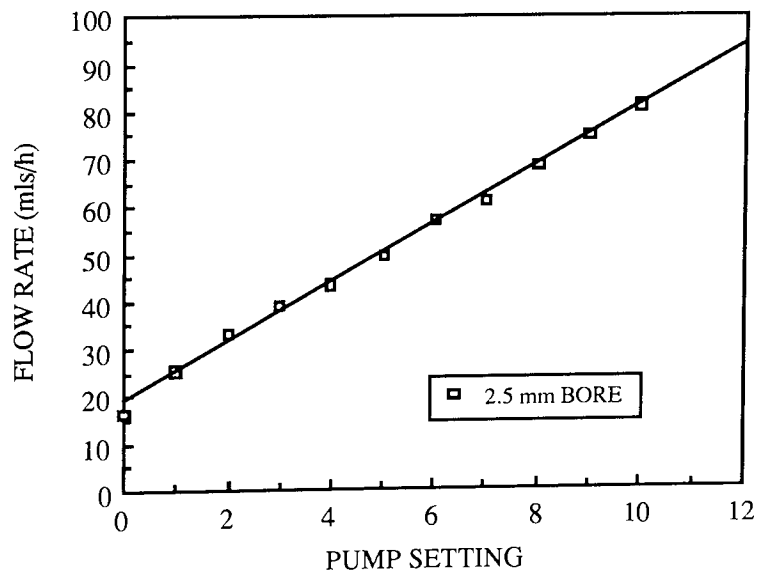


Fig 3.2.2.6



Flow rates obtained with the peristaltic pump with different tube bores.

Fig 3.2.2.7
Relationship between chemostat optical density
and dilution rate for *P. aeruginosa* PAO1.

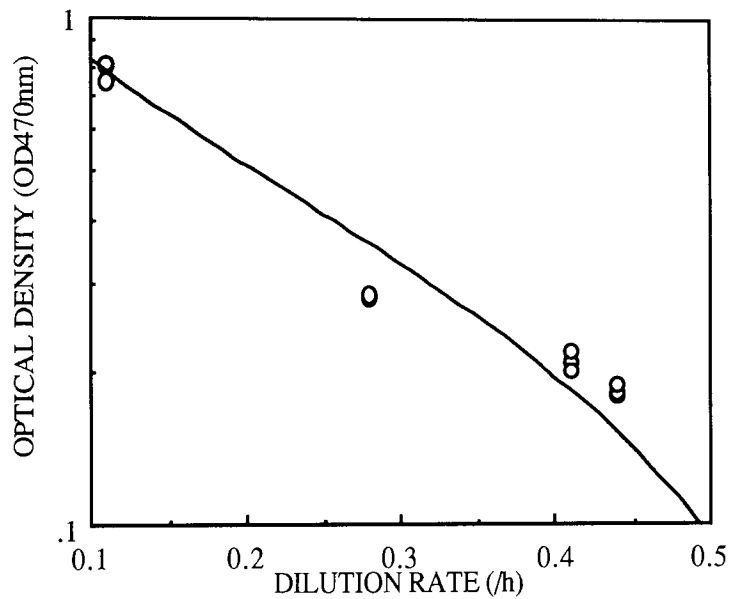
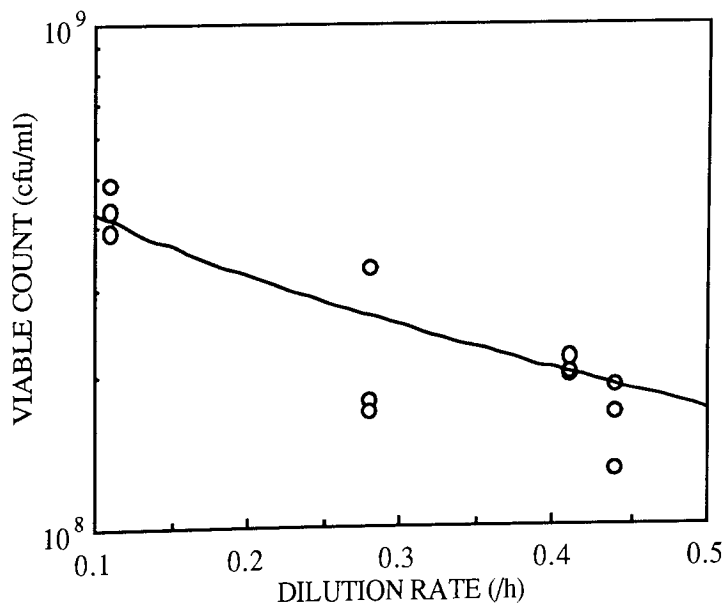


Fig 3.2.2.8
Relationship between chemostat viable count
and dilution rate for *P. aeruginosa* PAO1.



3.3 PREPARATIVE TECHNIQUES.

3.3.1 Preparation of bacterial outer membranes.

Outer membranes (OMs) were prepared by the method of Filip *et al.*, (1973). Cells were grown under various conditions at 37°C in an orbital shaking incubator and harvested by centrifugation at 5000 x g for 10min at 4°C. The resulting pellet was washed once in 0.85% saline and then suspended in 10ml double distilled water. Cells were broken by 10 x 30s pulses of sonication in an ice bath with 30s intervals for cooling. Sarkosyl (N-Lauryl Sarcosine) was added to a final concentration of 2%v/v to solubilise the cytoplasmic membrane and leave the OM intact (Lambert and Booth, 1982). After incubation at room temperature for 1h, any remaining unbroken cells were removed by centrifugation at 5000 x g for 10min. The supernatant was then centrifuged at 100,000 x g for 40min at 4°C. The final OM pellet was washed in distilled water and stored at -20°C.

3.3.2 Extraction and purification of lipopolysaccharide (LPS).

LPS was extracted by an adaptation of the hot phenol technique of Westphal and Jann (1965). Early stationary phase bacteria cultivated in 2L CDM were harvested by centrifugation at 5000 x g for 10min. The cells were washed once in 0.85% saline before being suspended in 20ml Tris-HCl (30mM pH8.0) and broken by 10 x 20s pulses of sonication in an ice bath with 30s intervals for cooling. Deoxyribonuclease (Bovine pancreas type III), ribonuclease (Bovine pancreas type 1-AS) and lysozyme were added at 0.1mg.ml⁻¹ to digest the DNA, RNA and peptidoglycan, respectively. After incubation for 2h at 37°C, 5ml tetrasodium EDTA (0.5M) and protease (*Strep. griseus* type XIV; final concentration 1mg.ml⁻¹) were added and the mixture incubated overnight at 37°C with constant shaking. The protease digests protein and EDTA chelates magnesium cations from the lipopolysaccharide, destroying its integrity. The protease is subsequently inactivated by heating to 80°C for 20min. The digested cell suspension was mixed with an equal volume of 90%w/v phenol, preheated to 80°C and stirred vigorously for 10min. The preparation was centrifuged at 5000 x g for 30min to permit phase separation and the upper layer containing LPS was carefully removed. Care was taken not to disturb any remaining

proteinaceous material at the interface. Two further extractions were performed by reheating the phenol layer to 80°C and adding a further 50ml distilled water at the same temperature. The aqueous extracts were pooled and dialysed for 48h against tap water to remove the phenol.

After dialysis $MgCl_2$ was added to a concentration of 50mM, which aids the formation of LPS micelles. The LPS was pelleted by centrifugation at 100,000 x g for 4h, washed once in double distilled water, centrifuged again and lyophilised.

3.3.3 Absorption of antisera with extracted LPS.

Sera were adsorbed with purified LPS from whole cells (section 3.3.2) to avoid immunological detection of proteins due to co-migrating LPS (Poxton *et al.*, 1985). Two mg of purified LPS was added to 1ml of serum and shaken at 37°C for 4h and then at 4°C for 24h. The serum was then centrifuged in an Eppendorf microcentrifuge for 30min to pellet the immune complexes. The procedure was repeated and the supernatant recovered and stored at -20°C.

3.3.4 Dry weight determinations.

Glass sample pots were allowed to equilibrate overnight on the freeze-dryer and their individual weights recorded. Cells were harvested by centrifugation at 10,000 x g for 10 min and the pellet washed twice with double-distilled water and resuspended to a determined OD_{470nm} of 4. Three 1ml aliquots were placed in separate sample pots and freeze-dried to constant weight.

3.4 ANALYTICAL TECHNIQUES.

3.4.1 Determination of the minimum inhibitory concentration of antibiotics.

Aliquots of double strength CDM (2.4ml) were dispensed into test-tubes with 0.5ml glucose (0.4M) and for CDM+Fe conditions 50µl iron (10mM). Amounts of sterile antibiotic solution and distilled water were added to give the desired concentration in a 2ml volume and finally a 100µl inoculum (diluted in CDM to give 1×10^6 cfu.ml⁻¹) from an overnight culture was added. The tubes were vortexed and incubated at 37°C for 18h. The tubes were examined for growth and the MIC defined as the lowest concentration of antibiotic that inhibited the development of visible growth.

3.4.2 Viable counts.

These were determined by using the spread plate method (Crone, 1984). Viable counts were performed on cell suspensions by preparing suitable serial dilutions in CDM salts (CDM without added glucose) to produce between 30-300 colonies per plate. Volumes of 100µl were plated in triplicate for each dilution on predried nutrient agar plates (NA). Plates were incubated at 37°C for 20h and colonies were enumerated using a colony counter. The viable count for the original suspension was established from the mean number of colony forming units (cfu) from a triplicate set of plates and multiplied by the dilution factor (cfu.ml⁻¹).

3.4.3 Antimicrobial bactericidal assay.

The 4-quinolones cause filamentation of bacteria which increases the turbidity of liquid cultures. Consequently bacterial killing by 4-quinolone antibacterials cannot be judged by measurements such as light scattering or optical density that merely reflect a change in bacterial mass. Hence, the rates of kill of bacteria by the 4-quinolones can be studied by viable counts (Smith, 1984b; section 3.4.2).

Two antimicrobial agents were chosen for this study, the 4-quinolone ciprofloxacin and the

aminoglycoside gentamicin. Stock solutions of each agent were prepared aseptically in sterile distilled water for bactericidal assays. These were stored at 4°C when not in use and replaced at weekly intervals.

Preliminary experiments were conducted to select concentrations of antimicrobial agents which gave appropriate levels of killing (1-2 log cycles), relative to control suspensions, within a 1h contact time period at 37°C. There was no sign of the paradoxical dose-dependent responses reported previously for ciprofloxacin (Crumplin and Smith, 1975; Smith, 1986b). Instead levels of survival in all cases decreased with increasing drug concentration (Evans *et al.*, 1991; Fig 4.7.1 and 4.7.2). Perhaps if higher drug concentrations were used this biphasic response would be seen.

Tubes containing appropriate concentrations of antimicrobial agents (ciprofloxacin concentrations from 0-5µg.ml⁻¹ and gentamicin 0-20µg.ml⁻¹) in CDM salts solution, were prepared and pre-warmed at 37°C. Cultures of *P. aeruginosa* PAO1 at various stages in the growth curve in batch culture and at various growth rates in the chemostat were standardised to an OD_{470nm} of 0.1 in CDM salts solution. Aliquots of this suspension (100µl) were added to the antimicrobial solutions to give the desired concentration and control tubes (9.9ml) at staggered time intervals (1 min) and incubated at 37°C for 1h. This achieved a cell density of 1x 10⁶ cfu.ml⁻¹ (Table 3.4.3).

Further serial dilutions (100µl) were made in sterile CDM salts solution (0.9ml and 9.9ml) to dilute out the antimicrobial agents with the minimal of time delay and plated on NA in triplicate (section 3.4.2). All plates were subsequently incubated for 20h at 37°C. Results are expressed as the logarithm of the percentage reductions in viability relative to appropriate unexposed controls. Each experiment was repeated twice for each cell suspension with each antimicrobial agent. Control experiments in the absence of the drug performed at the beginning and the end, showed there to be little multiplication of the cells during the experimental procedure for any of the inocula. This procedure for assessing bactericidal activity did not include washing or centrifugation steps and allowed the cells to be challenged immediately after leaving either batch or chemostat growth conditions.

Table 3.4.3 Antimicrobial solutions used in bactericidal assays.

Antimicrobial stock soln.	Volume used (μl)	CDM salts soln. (ml)	Inoculum (μl)	Final conc ($\mu\text{g.ml}^{-1}$)
Ciprofloxacin $20\mu\text{g.ml}^{-1}$	—	9.9	100	control
	25	9.875	100	0.05
	50	9.85	100	0.1
	125	9.775	100	0.25
	250	9.65	100	0.5
	500	9.4	100	1.0
	1250	8.65	100	2.5
	2500	7.4	100	5.0

Gentamicin $100\mu\text{g.ml}^{-1}$	—	9.9	100	control
	100	9.8	100	1.0
	200	9.7	100	2.0
	400	9.5	100	4.0
	500	9.4	100	5.0
	1000	8.9	100	10.0
	2000	7.9	100	20.0

3.4.4 Selection of resistance.

Serial subculture of *P. aeruginosa* PAO1 in the presence of increasing concentrations of ciprofloxacin resulted in rapid selection of resistance, producing cells which were highly resistant to ciprofloxacin.

P. aeruginosa PAO1 grown under iron-deprived or iron-plentiful conditions were inoculated into increasing sub-inhibitory concentrations of ciprofloxacin, starting with an inoculum of 1×10^6 cfu.ml⁻¹. After 48h incubation at 37°C the tube showing turbidity at the highest

concentration of ciprofloxacin was subcultured into a new dilution series. This was repeated until 2, 10, 20 and 30 x the original MIC was obtained (Fig 4.9).

3.4.5 Fatty acid analysis.

Alkaline hydrolysis may be used to extract fatty acids, ester linked to cellular phospholipids from whole cells. However, extracted LPS contains fatty acids which are amide-linked to the diglucosamine disaccharide molecules in lipid A and subsequently require the more severe acid hydrolysis to release them. Free fatty acids were analysed by gas liquid chromatography.

3.4.5.1 Alkaline hydrolysis of whole cells.

Fatty acids were extracted according to the method of Moss (1978). Whole cells of *P. aeruginosa* grown to early stationary phase in CDM+Fe and CDM-Fe with, and without sub-MICs of Mitomycin C (see section 5.7.2), were harvested by centrifugation at 5000 g for 10min, washed once in saline and suspended in saline to OD_{470nm} 5.0. To each 0.5ml cell suspension a known volume of internal standard (C_{17.0} heptadecanoic acid) was added since it was not present as a fatty acid in *P. aeruginosa*. The preparations were then placed in sealed Pyrex hydrolysis tubes with teflon-lined caps (Sterilin, Teddington, Middlesex), previously cleaned by soaking overnight in EDTA 0.01%w/v at room temperature, followed by 1M H₂SO₄. The cells were mixed with 5% NaOH/50% aqueous methanol (2.5ml), the tubes sealed and heated at 100°C for 30min. On cooling the contents were adjusted to pH2 with concentrated HCl. The addition of 2.5ml boron trifluoride-methanol complex (14%w/v boron trifluoride) followed by heating at 80°C for 5min, produced the methyl esters of the free fatty acids. These were extracted from the preparation with 5ml chloroform/hexane (1:4). The upper organic layer, containing the fatty acid methyl esters, was removed and evaporated to dryness under vacuum in a rotary evaporator. The residue was dissolved in 40µl hexane and allowed to evaporate at room temperature to a final volume of approximately 10µl. Control tubes, to which bacteria were not added, were also prepared.

3.4.5.2 Acid hydrolysis of whole cells.

Whole cells, as described above, plus the internal standard were placed in sealable tubes and hydrolysed by boiling in 6M HCl for 4h. After cooling the methyl esters were formed by the addition of 2.5ml boron trifluoride (14%w/v). The fatty acids were then extracted as for alkaline hydrolysis.

3.4.6 Gas liquid chromatography (GLC).

Free fatty acid methyl esters were analysed by loading (2 μ l) onto a GLC column packed with 3% SP-2100 DOH on 100/120 supelcort (Supelco Chromatography Supplies, Supelchem, Sawbridgeworth, Herts.). The column was operated under the following conditions:

Column temperature	150°C
Final temperature	225°C
Rate of increase	2°C.min ⁻¹
Injector temperature	200°C
Detector temperature	300°C
Initial time	5min
Final time	10min
Gas pressures: Hydrogen	14.5psi (for flame ionisation detector)
Air	6.5psi (for flame ionisation detector)
Nitrogen	15.0psi (as carrier gas through column)

An external standard of bacterial acid methyl ester mix (10mg.ml⁻¹) (Supelco Chromatography Supplies, Supelchem, Sawbridgeworth, Herts.), containing 23 fatty acids was loaded (1 μ l) to quantitate the GLC analysis. Thus, *P. aeruginosa* fatty acids could be identified by comparing retention times with this standard. The integration readings were calculated for each peak and quantified by comparison with the value of the internal standard (C_{17:0} heptadecanoic acid).

3.4.7 Lowry Protein Assay.

The protein content of OM preparations was determined by the method of Lowry *et al.*, (1951) and simplified according to Peterson (1977). Bovine serum albumin (BSA) standards (0-300 μ g) and the samples for analysis were made up to 0.5ml with double distilled water. An equal volume of 1M NaOH was added to each sample and heated to 100°C for 5min. On cooling 2.5ml of a solution containing 1ml 0.5%w/v $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 1ml 1%w/v NaK tartrate solution in 50ml of 5%w/v Na_2CO_3 in 0.1M NaOH was added. After leaving for 10min, 0.5ml Folin-Ciocalteau reagent diluted 1:1 with distilled water was added to each sample and mixed by vortexing. The $\text{OD}_{750\text{nm}}$ was recorded after 30min against a BSA blank and a calibration curve constructed (Fig 3.4.7). Standards and samples were assayed in triplicate.

3.4.8 2-Keto 3-deoxy-D-manno-2-octulosonic acid (KDO) assay.

The KDO content of OM preparations and extracted LPS samples were measured using a method based on that of Karkhanis *et al.*, (1978). This was chosen in preference to the method of Osborn (1963), since the development of turbidity in the reaction mixture makes the optical density difficult to read and is therefore a major disadvantage. Samples of KDO standard (2 to 50 μ g.ml⁻¹) or 2mg of LPS containing material or alternatively 50 μ l and 100 μ l of OMP preparation (which can be standardised by relating to the protein content, see section 3.4.7) were made up to 1ml with 0.1M H_2SO_4 and hydrolysed by heating in sealable tubes at 100°C for 30min to completely release the KDO. On cooling, the preparations were centrifuged in an Eppendorf microcentrifuge for 5min to eliminate interference due to insoluble material and the upper clear layer removed. A volume of 0.25ml periodic acid (0.04M in 0.0625M H_2SO_4) was added and the contents were mixed thoroughly before being allowed to stand at room temperature for 20min. This was followed by the addition of 0.25ml sodium arsenite (2.6% in 0.5M HCl) and the contents mixed thoroughly. After the brown colour had disappeared 0.5ml thiobarbituric acid (0.6%w/v) was added, the tubes were sealed and heated to 100°C for 15min. While hot, 1ml of dimethyl sulphoxide was added to prevent the formation of turbidity and the contents allowed to cool. The absorbance of each solution at 548nm was then read against a blank treated as above

Fig 3.4.7

Calibration curve for the estimation of protein.

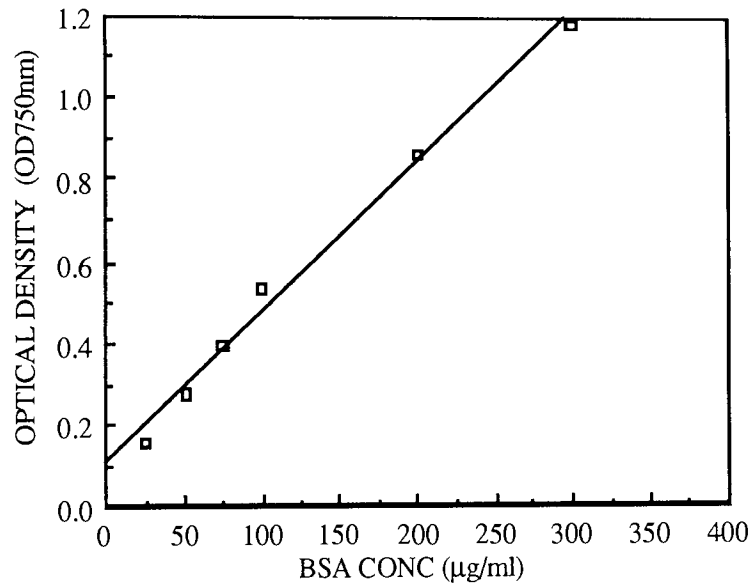
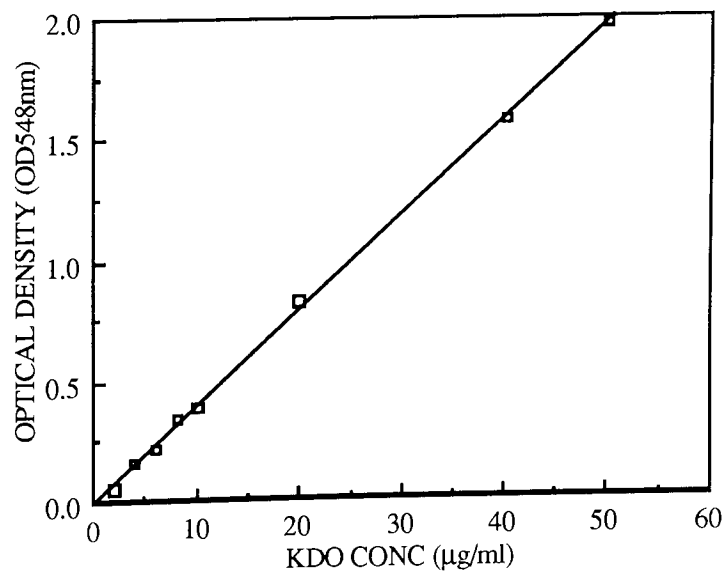


Fig 3.4.8

Calibration curve for the estimation of
2-Keto 3-deoxy-D-manno-2-octulosonic acid (KDO).



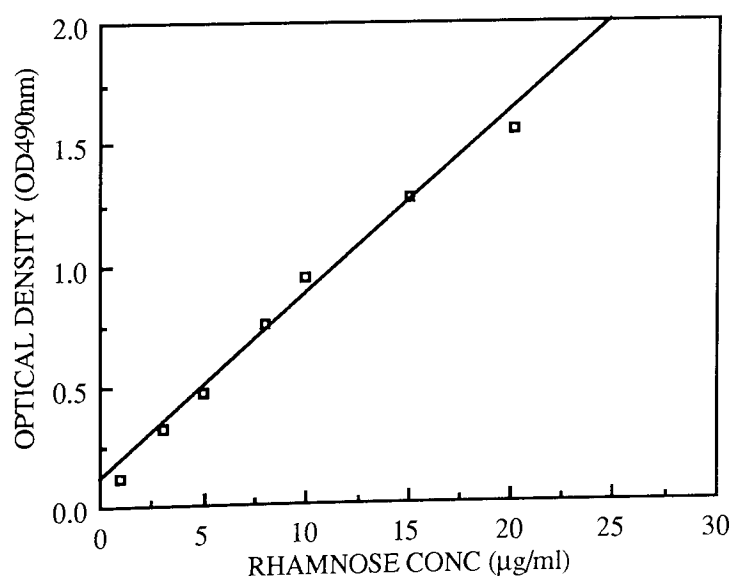
without KDO. Standards and samples were assayed in triplicate. By plotting a standard calibration curve of KDO concentration against OD_{548nm} the KDO content was calculated (Fig 3.4.8).

3.4.9 Phenol-Sulphuric acid assay.

The method used was based on that of Dubois *et al.*, (1956). Samples of rhamnose standard (1.0 to $20\mu\text{g}\cdot\text{ml}^{-1}$) or $100\mu\text{l}$ of OMP preparations at several dilutions (which can be compared by relating to the protein content; see section 3.4.7), were standardised to a total volume of $100\mu\text{l}$ with double distilled water. To each preparation $100\mu\text{l}$ of phenol (5%w/v) and $500\mu\text{l}$ 1M H_2SO_4 was added. The contents were mixed thoroughly and allowed to stand at room temperature for 15min. The OD was read at 490nm . A calibration curve was constructed of rhamnose concentration against OD_{490nm} to establish the carbohydrate content of the O-side chain of LPS. Standards and samples were assayed in triplicate and all glassware was acid washed with 1M HNO_3 (Fig 3.4.9).

Fig 3.4.9

Calibration curve for the estimation of carbohydrate.



3.4.10 Bacterial adherence to hydrocarbon (BATH).

The technique used was developed by Rosenberg *et al.*, (1980), and modified according to Williams *et al.*, (1986). It is designed to assay the degree of hydrophobicity of bacterial cells based on the partitioning of cells between two immiscible liquid phases.

Since adherence of a given strain may depend largely on the growth conditions employed, cells were taken at specific phases of growth. The OD_{470nm} of early stationary phase cells grown in CDM+Fe and CDM-Fe were measured. The cells were centrifuged at 5000 x g for 10min, washed once in PUM buffer and resuspended to an OD of 0.3.

PUM buffer:	K ₂ HPO ₄ .3H ₂ O	22.2g
	KH ₂ PO ₄	7.26g
	Urea	1.8g
	MgSO ₄ .7H ₂ O	0.2g
	Distilled water to	1L
	pH 7.1	

The washed bacterial suspensions were vortexed under controllable conditions for 2min with the aliphatic hydrocarbon hexadecane (250μl) and left at room temperature for 20min. After complete separation of the phases, 1ml of the lower aqueous phase was removed and the OD_{470nm} measured. Hexadecane was used in this study as it is the hydrocarbon of choice as the organic test fluid in the BATH assay (Vanhaecke and Pijck, 1988).

More hydrophobic bacteria will bind to the organic droplets following the mixing procedure, bringing about a concomitant drop in the cell density of the bulk aqueous phase. The results were then recorded as the percentage absorbance of the aqueous phase after treatment, relative to the initial absorbance of the bacterial suspension. Each value represents the mean of 3 independent determinations.

The assay can be overloaded if the volume of hydrocarbon is too low or the initial bacterial suspension is too concentrated. Therefore, the volume of hydrocarbon used in this study

was determined by adding 1.5ml of a bacterial suspension OD of 0.3 to increasing volumes of hexadecane. A calibration curve was constructed (Fig 3.4.10.1) of OD_{470nm} against volume of hydrocarbon and used to establish the optimal volume of 500µl.

Insufficient mixing may not allow for maximal bacterial adherence. This can be easily ascertained by constructing a calibration curve of the increase in duration of the vortexing procedure (Fig 3.4.10.2) with absorbance. Two minute vortexing was adequate for each sample. All glassware was acid washed with 1M HNO₃.

3.4.11 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Separation of OM proteins was carried out by gel electrophoresis using the Mini-Protean system and a large slab gel apparatus, according to the methods described by Lugtenberg *et al.*, (1975), as modified by Anwar *et al.*, (1983b).

The running and stacking gel were prepared as described in table 3.4.11 (larger quantities were used for the slab gels) and polymerisation was initiated by the addition of N,N,N,'N'-tetramethylethylene diamine (TEMED). The running gel was poured between the glass plates separated by 0.5mm (Mini-Protean) or 1mm (slab gel) plastic spacers and allowed to set for 10min. A spray of electrode buffer on top of the gel ensured complete polymerisation. This buffer solution was removed and the stacking gel was cast in a similar manner. A teflon comb was inserted between the plates to create wells for sample application.

Samples were denatured at 100°C for 10min with an equal volume of sample buffer (table 3.4.11) before loading onto the gel (1mg.ml⁻¹, section 3.4.7). The electrode buffer contained 0.025M Tris, 0.19M glycine and 0.1%w/v SDS. In the Mini-Protean system a constant voltage of 200V was applied across the gel and electrophoresis continued until the tracking dye had migrated to within 0.5cm of the bottom of the gel. For large slab gel systems a constant current of 40mA was applied and ran until the tracking dye had moved approximately 12cm.

Fig 3.4.10.1

Calibration curve to determine the optimum volume of hexadecane for *P. aeruginosa*.

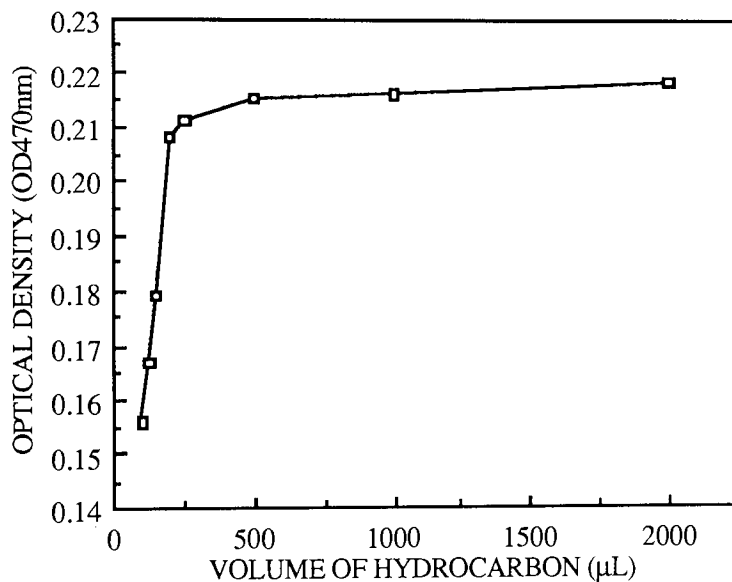
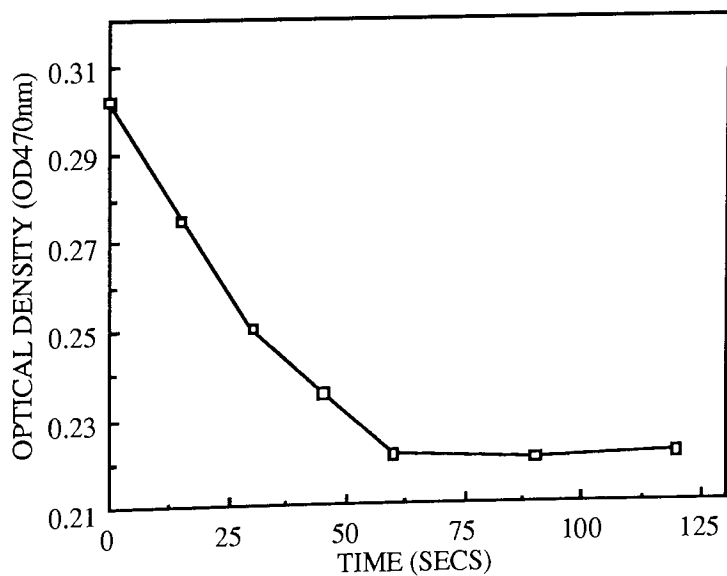


Fig 3.4.10.2

Adherence of *P. aeruginosa* to hexadecane as a function of duration of mixing.



Gels were either used directly for immunoblotting (section 3.5.1) or stained for protein by staining the gel with 0.1%w/v Coomassie brilliant blue R-250 in 50% methanol/10% acetic acid for 60min. Gels were subsequently destained in 10% methanol/20% acetic acid and photographed using diffuse transmitted light and finally dried onto filter paper using a gel dryer. Coomassie blue stained gels were scanned with an LKB 2202 Ultrascan Laser densitometer run by an Apple II Europlus computer. This was repeated on different gels and an average value taken.

Denatured polypeptides bind SDS in a constant ratio and have essentially identical charge densities and therefore migrate in polyacrylamide gels according to their size. Molecular weights of OMPs separated by SDS-PAGE can be predicted by comparison with standard marker proteins. Pre-stained molecular weight markers (Daltons) were obtained from Bio-Rad which consisted of:

Phosphorylase B	110,000
Bovine serum albumin	84,000
Ovalbumin	47,000
Carbonic anhydrase	33,000
Soybean trypsin inhibitor	24,000
Lysozyme	16,000

Table 3.4.11 Composition of running gel, stacking gel and sample buffer for SDS-PAGE.

CONSTITUENT	RUNNING GEL		STACKING GEL	SAMPLE BUFFER
	12%	14%		
stock 1	5.25ml	6.25ml	_____	_____
stock 2	_____	_____	1.7ml	_____
1.5M Tris ¹ pH8.8	6.25ml	6.25ml	_____	_____
0.5M Tris ¹ pH6.8	_____	_____	2.5ml	2.5ml
10%w/v SDS	0.5ml	0.5ml	0.1ml	5.0ml
distilled water	7.16ml	6.16ml	5.3ml	5.0ml
10%w/v APS ²	70µl	70µl	30µl	_____
TEMED ³	45µl	45µl	25µl	_____
2-mercapto- ethanol	_____	_____	_____	0.5ml
5% bromophenol blue	_____	_____	_____	0.4ml
glycerol	_____	_____	_____	2.5ml

stock 1 = 44%w/v acrylamide and 0.8%w/v N,N'-methylene-bis-acrylamide (BIS)

stock 2 = 30%w/v acrylamide and 0.8%w/v BIS

¹Tris (hydroxymethyl) aminoethane

² Ammonium persulphate (freshly prepared)

³ N,N,N,'N'-Tetramethylethylene diamine

3.4.12 Gel electrophoresis of lipopolysaccharide (LPS).

LPS was separated by SDS-PAGE using 12% acrylamide gels and the buffer system described for gel electrophoresis of OMPs (section 3.4.11). Proteinase K digests of either bacterial OM preparations or whole cells were analysed, based on the method of Hitchcock and Brown (1983). One hundred μl of whole cells ($\text{OD}_{470\text{nm}}$ 10) or OM (equivalent to $1\text{mg}\cdot\text{ml}^{-1}$ protein) were denatured at 100°C for 10min with $80\mu\text{l}$ sample buffer (table 3.4.11). On cooling $20\mu\text{l}$ of sample buffer containing proteinase K ($2.5\text{mg}\cdot\text{ml}^{-1}$) was added and the preparation incubated at 60°C for 1h. Samples were loaded ($10\mu\text{l}$ for whole cells) onto the acrylamide gels as for OMPs and electrophoresis carried out at a constant voltage of 100V for small gels and a constant current of 25mA for the large slab gels. Gels were either used directly for immunoblotting (section 3.5.1) or stained with silver (section 3.4.13).

3.4.13 Silver stain of LPS.

LPS separated by SDS-PAGE was visualised by silver staining following the method of Tsai and Frasch (1982). After electrophoresis the gel was immersed overnight in 40% ethanol/5% acetic acid solution. This fixing solution was then replaced with fresh solution containing 1% periodic acid and the LPS oxidised for 1h. After 3 x 30min washes with double distilled water to remove any unreacted periodic acid, staining reagent was poured over the gel. The silver stain was freshly prepared by slowly adding 5ml of 20%w/v silver nitrate solution to a mixture of 2ml concentrated ammonium hydroxide and 28ml 0.1M NaOH. The solution was made up to 150ml with double distilled water and the gel shaken for 45min. After further washing was performed for 1h with two changes of water, a developing solution consisting of 25mg citric acid, 0.5ml formaldehyde (37%) in 500ml double distilled water was added to the gel.

When the LPS was stained to the desired intensity (5 to 20min), the colour development was terminated by replacing the developer with fixing solution. Gels were photographed immediately. Staining and washing times were reduced to 30min for gels run on the Mini-Protean system.

3.4.14 Determination of serum iron concentration.

Measurement of the concentration of iron in the serum of leukaemic patients taken before the onset of infection, throughout the infection episode and on recovery, provides a useful method to assess both 'free' and protein-bound iron during infection in neoplastic disease. The principle of the method is based on that of Williams and Conrad (1966) and involves the separation of Fe^{3+} from transferrin by means of a detergent mixture (sodium acetate) in a slightly acidic solution. On addition of the chromogen (ferrozine) a coloured complex is formed and its absorbance measured at a wavelength of 562nm (Carter, 1971; Sidel *et al.*, 1984).

The reagent was freshly prepared by adding 39mg of ascorbic acid to 10ml buffer (sodium acetate detergent mixture, 170mmol.L⁻¹, pH 5.5; Boehringer Mannheim, West Lothian, U.K.). A 150 μ l volume of chromogen (ferrozine, 80mmol.L⁻¹) was added to 600 μ l of double distilled water to produce the start reagent. Using a Cobas bio Centrifugal analyser (Roche), the reagent boat was filled with the ascorbic acid reagent and the chromogen reagent put in the start reagent well. An iron solution of known concentration was placed in the standard containers (Boehringer Mannheim, West Lothian, U.K.), which was treated in the same way as the serum samples and provided reference values for the assay. Finally all serum specimens were loaded. The Centrifugal analyser automatically mixes the samples/controls with the reagents, pipetting them into iron-free reaction vessels. Incubation then takes place for 5min at 37°C and the OD_{562nm} of the samples and controls are read within 30min.

A reagent blank must be determined once for each series by running the assay using double distilled water instead of sample material. The absorbance difference thus obtained is subtracted from the absorbance difference obtained for each sample ($A_{\text{sample}} - A_{\text{sample blank}}$). The reference ranges for male and females (Microbiology Department, Queen Elizabeth Hospital) are:

male: 10-32 μ mol.L⁻¹

female: 5-30 μ mol.L⁻¹

3.4.15 Determination of serum transferrin level.

Transferrin is the principle protein for iron transport and its concentration correlates with the total iron binding capacity. Evaluation of plasma transferrin levels were used to assess the effect of infection in the leukaemic patient. This was achieved by measuring transferrin levels in serum samples taken prior, during and on recovery from an infection episode. The principle of the method was based on that of Leonberg *et al.*, (1987). The antibody reagent set for serum proteins (SPQ; Atlantic Antibodies, an Incstar company, Maine, U.S.A.), permits the quantitative determination of transferrin by immunoturbidimetry. That is the quantification of antigen by the addition of antibody, to form antigen-antibody complexes. The complexes scatter light and reduce the measured light in proportion to their concentration.

A reagent blank must be determined by running the assay using double distilled water (control). Standards (containing known concentrations of transferrin), controls/patient serum samples were diluted 1:26 with saline diluent.

The antibody (goat anti-human transferrin) is warmed to room temperature and gently mixed before use. Microvolumes of these prediluted samples and the antibody are pipetted into a disposable cuvette rotor. The sample (antigen) and antiserum solutions were then mixed in the reaction cuvettes by the Cobas bio Centrifugal analyser. Insoluble antigen-antibody complexes begin to form immediately after mixing. These complexes produce a reduction in transmitted light which is measured by an increased absorbance at OD_{340nm} .

A calibration curve is generated by assaying a series of 5 standards with known concentrations of specific protein and plotting the change in absorbance versus concentration. Concentrations for the control and patient samples are interpolated from the calibration curve. The reference range for transferrin is 2- 3.6g.L⁻¹ (Finch and Huebers, 1982).

3.5 IMMUNOLOGICAL TECHNIQUES.

3.5.1 Immunoblotting.

The transfer of OM components, separated by SDS-PAGE, to a nitrocellulose (NC) membrane for immunoreaction was performed following the method of Towbin *et al.*, (1979), as modified by Anwar *et al.*, (1984). Following electrophoresis the gel and NC (Trans Blot Membrane, pore size 0.45µm, Bio-Rad Laboratories Ltd., Watford, Herts.), were sandwiched between chromatography paper (Whatman Ltd., Maidstone, Kent), and Scotchbrite pads (Bio-Rad Laboratories Ltd., Watford, Herts.), in a Trans Blot cassette. Electroblothing was performed in a Trans Blot cell at 100V for 1h filled with transfer buffer (192mM glycine, 25mM Tris, 20% methanol, pH 8.3), at 4°C. Efficiency of protein transfer is known to be a function of molecular weight (Burnette, 1981) and these conditions were necessary to ensure complete transfer of high molecular weight proteins. Qualitative transfer of OMPs was confirmed by staining with 1%w/v amido black in 10% methanol/20% acetic acid or alternatively by staining the gel after blotting with coomassie blue (section 3.4.11). LPS separated by SDS-PAGE was transferred to NC at 50V for 1h.

After transfer the NC paper was soaked in Tris buffered saline (TBS; 10mM Tris HCl, 0.9%w/v NaCl, pH7.4) containing 0.3%v/v Tween-20 (TBS/Tween) for 1h to saturate non-specific binding sites on the NC (Batteiger *et al.*, 1982). The NC was incubated for 4h at 37°C in patient serum, diluted 1 in 25 in TBS-Tween. Following this the immunoblot was washed in TBS and incubated for a further 2h at 37°C with either protein A horseradish peroxidase (HRP) conjugate, goat anti-human IgG (0.25µg.ml⁻¹; Miles Scientific, Rehovot, Israel) or protein A alkaline phosphatase conjugate IgG (AP; Sigma), diluted 1 in 1000 in TBS-Tween.

After further washing in TBS, the HRP conjugates were visualised using a solution containing 25µg.ml⁻¹, 4-chloro-1-naphthol and 0.01%v/v hydrogen peroxide in TBS. On reaching the desired intensity the reaction was terminated by placing the immunoblot in double distilled water.

The antigenic sites for AP were visualised by adding a developing solution containing 33 μ l NBT (Nitro blue tetrazolium 50mg.ml⁻¹ in 70% dimethyl formamide)/5ml of alkaline phosphate buffer (100mM Tris-HCl, 100mM NaCl, 5mM MgCl₂, pH9.5). The colour reaction was allowed to develop and the reaction terminated by placing the immunoblot into a solution containing 20mM Tris-HCl and 0.5mM EDTA, pH8.0 (Blake *et al.*, 1984). The immunoblots were stored dry.

The reaction of replicate antigenic patterns probed with patient serum taken throughout an infection episode was investigated by performing strip immunoblots. A comb with one large well was inserted into the stacking gel and after electrophoretic transfer, the NC paper was divided into 5mm strips and incubated in serum, as described above.

3.5.2 Enzyme-linked immunosorbent assay (ELISA).

A whole cell ELISA method was performed according to procedure of Borowski *et al.*, (1984) for detection of IgG, IgA and IgM antibodies directed against antigens of various clinical isolates of *P. aeruginosa*, *E. coli* and *K. pneumoniae*. Immulon 2 ELISA plates (Dynatech, Billinghamurst, Sussex), were coated with whole cells (200 μ l) which had been harvested by centrifugation at 5,000 x g for 10min at 4°C and suspended in PBS (NaCl 8%, KH₂PO₄ 0.2%, Na₂HPO₄.2H₂O 2.9%, KCl 0.2%, pH7.4), to OD_{470nm} 0.05. The plates were shaken for 30min at room temperature and left overnight at 4°C, so that the antigen would adhere to the plastic. The plates were washed 3 times with PBS containing 0.05% Tween 20. Non-specific binding sites were blocked with a blanking solution containing 10% foetal calf serum (Gibco, Ltd.), and 1% Bovine serum albumin in PBS by shaking at room temperature for 30min and then 2h at 37°C. The plates were washed as above and 100 μ l of the serum under investigation, serially diluted in gelatin phosphate buffer (0.07M sodium phosphate buffer pH6.5 containing 0.2%w/v gelatin) was added to each well and incubated for 2h with shaking at room temperature. Two rows of wells of each plate were incubated with plain gelatin phosphate buffer as a blank. The plates were again washed as above and incubated for 2h at 37°C with 100 μ l of 1 in 1000 dilution of conjugated antibody in blanking solution per well (IgG, IgA and IgM anti-human peroxidase conjugates were used in this study; Sigma).

Following a further wash, 100µl of substrate was added to each well. The substrate solution was prepared by dissolving 10mg 3,3',5,5'-tetramethylbenzidine (TMB) in 1ml dimethylsulphoxide and making it up to 100ml with 0.1M sodium acetate/citrate buffer pH6. Immediately before use 8µl hydrogen peroxide (6%v/v) was added and the plates were shaken at room temperature until a blue colour developed (approximately 10min). The reaction was stopped by the addition of 50µl of 1M H₂SO₄ to each well and a yellow colour developed. The absorbance of each well was measured at OD_{450nm} using a Dynatech plate reader.

The mean absorbance of the control wells containing gelatin phosphate buffer and conjugate were subtracted from the test wells. The ELISA titer was taken as the dilution at a net absorbance of 0.1. All ELISA determinations were performed in duplicate.

RESULTS CHAPTER 4

4. EFFECT OF IRON CONCENTRATION AND GROWTH RATE/PHASE ON THE EXPRESSION OF PROTEIN G (OprG) IN *P. aeruginosa* : CORRELATION WITH ANTIBIOTIC SENSITIVITY.

4.1 INTRODUCTION.

In Gram-negative bacteria, it is the cell envelope which is the prime structure through which a bacterium interacts with a potentially changing environment. Consequently it has been found to exhibit a high degree of plasticity (Brown, 1977; Costerton *et al.*, 1979; Brown and Williams, 1985b). An example is the adaptation to low iron environments, particularly important for commensal organisms and potential pathogens, since free iron concentrations in body tissue fluids and secretions are extremely low (Neilands, 1982; Weinberg, 1984). Iron is of such importance to the cell that many species of bacteria have developed both high- and low-affinity specific uptake-systems (Weinberg, 1978; Griffiths, 1987b). These are regulated depending on the abundance of iron in the cell's environment (Griffiths, 1987b). The high-affinity systems, which are expressed under iron-restricted conditions, usually involve the export from the cell of iron-chelating compounds called siderophores. After binding to a molecule of ferric iron, the ferric-siderophore complex is then delivered to the bacterial cell by way of concomitantly produced iron-regulated membrane proteins (IRMPs; Weinberg, 1978; Bagg and Neilands, 1987). Little is known of the low-affinity pathways, but since deletion of the high-affinity system is not lethal and impairs growth only under severely iron-restricted conditions (e.g as found *in vivo*), the process must be widely distributed (Neilands, 1984; Griffiths, 1987b). This is demonstrated by the ability of siderophore-defective mutants of *Salmonella typhimurium*, to continue to grow maximally under iron-replete conditions (Pollack *et al.*, 1970). In addition, the fact that the high-affinity pathways are best understood at present does not necessarily mean that they are the most common, nor indeed the most efficient mechanism for assimilating iron. It may merely reflect the relative ease with which such mechanisms can be studied.

Environmental factors other than nutrients have also been found to exert changes upon

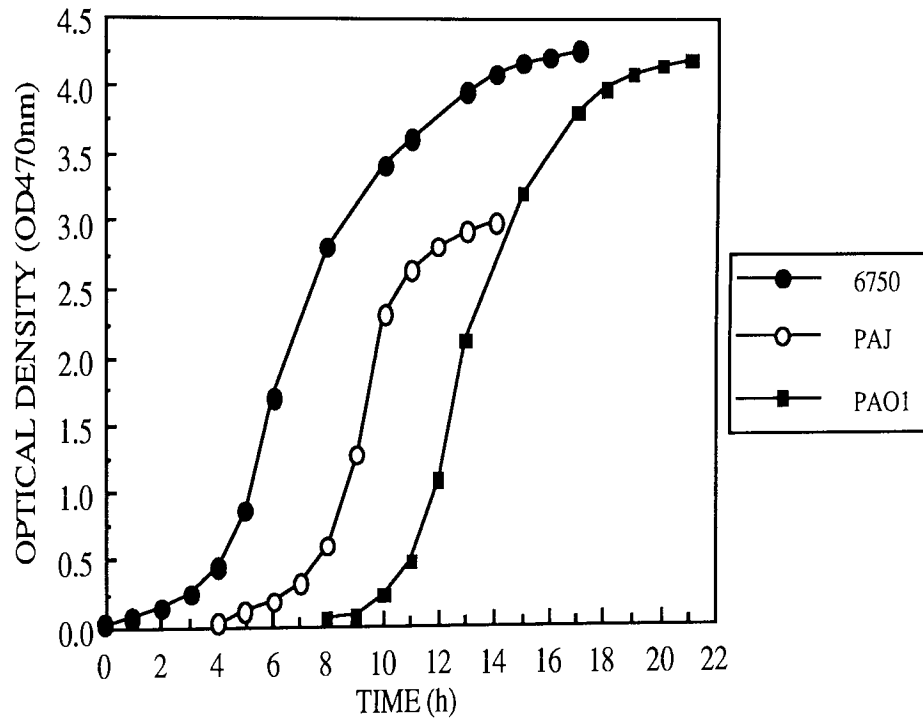
various components of the cell envelope. Growth rate may affect lipopolysaccharide (LPS) composition (Dodds *et al.*, 1987), whilst the phase of growth has been shown to affect the composition of the polysaccharide capsule (Annison and Couperwhite, 1987). Similarly temperature (Kropinski *et al.*, 1987) and medium osmolarity can bring about changes in the OMP profile (Kawaji *et al.*, 1979). Such phenotypic variation in OM composition of bacteria can affect susceptibility to antibiotics, disinfectants and preservatives as well as to host defences (Brown and Williams, 1985a; Gilbert *et al.*, 1987).

There is at present a paucity of data relating to protein G (OprG) of *Pseudomonas aeruginosa*, yet under certain conditions it can become a very prominent protein within the OM, although no function has yet been ascribed to it at present. The aim of this investigation was to characterise further OprG, by observing the effects of environmental factors upon its expression, the relative contributions of growth rate/phase and its potential contribution to susceptibility or resistance to antimicrobial agents.

4.2 GROWTH OF *P. aeruginosa* UNDER IRON-PLENTIFUL AND IRON-DEPLETED CONDITIONS.

The growth characteristics of *P. aeruginosa* strains 6750, PAJ and PAO1 in iron-plentiful and iron-depleted CDM in batch culture, were investigated by monitoring the optical density (OD) of the cultures at 470nm. The resulting growth curves are shown for CDM+Fe (Fig 4.2.1) and CDM-Fe (Fig 4.2.2). The doubling time and the growth rate of strains 6750, PAJ and PAO1 during the exponential phase was 72min, 1.2h⁻¹; 72min, 1.2h⁻¹; and 60min, 1h⁻¹, respectively. The growth rate of each of these strains was not affected by the concentration of iron in the medium. Onset of stationary phase occurred at lower optical densities and the overall bacterial yield was reduced in iron-depleted cultures compared to iron-plentiful media. Early stationary phase cells were taken at specific points in the growth curve, for 6750 OD= 3.75 in CDM+Fe compared to 1.2 in CDM-Fe; and for PAJ OD= 2.5 in CDM+Fe compared to 0.8 in CDM-Fe and for PAO1 OD= 3.5 in CDM+Fe as compared to 0.85 in CDM-Fe.

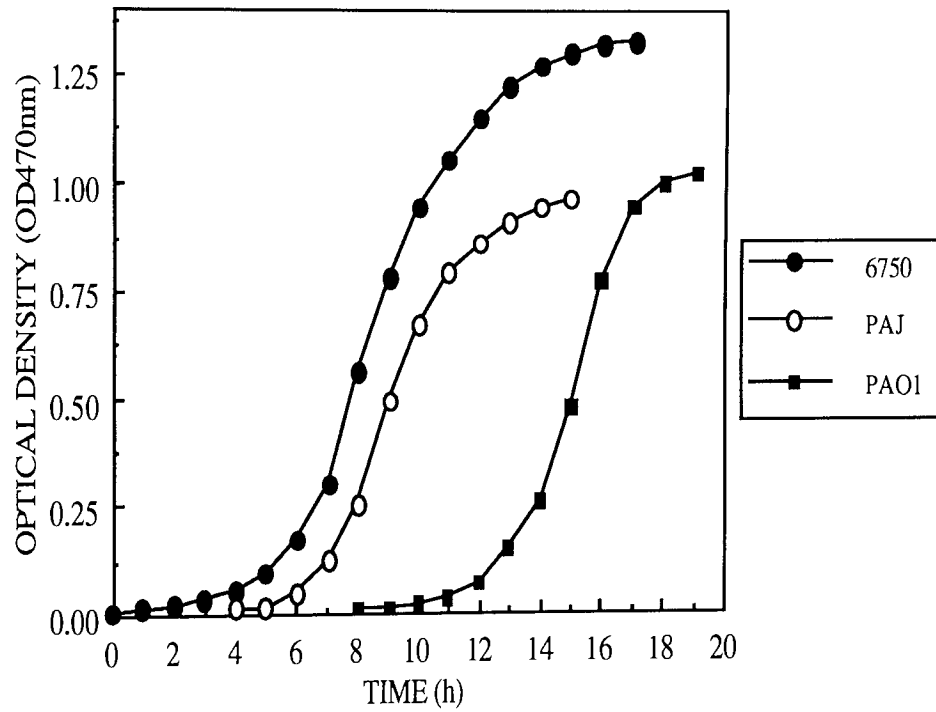
Fig 4.2.1
Growth of *P. aeruginosa* strains 6750, PAJ and
PAO1 in iron-plentiful CDM batch culture.



The curves have been offset for clarity.

Fig 4.2.2

Growth of *P. aeruginosa* strains 6750, PAJ and PAO1 in iron-depleted CDM batch culture.



The curves have been offset for clarity.

In iron-sufficient conditions, if all essential nutrients are present in excess the cells stop growing exponentially either when secondary metabolites in the culture medium reach toxic levels, or as a result of inadequate oxygenation. In iron-depleted media, iron, essential for many metabolic processes, becomes the limiting nutrient before secondary metabolites accumulate sufficiently to prevent growth. Hence the bacterial population is reduced.

4.3 ANALYSIS OF OUTER MEMBRANE PROTEIN (OMP) PROFILES OF *P. aeruginosa* CULTIVATED UNDER IRON-PLENTIFUL AND IRON-DEPLETED CONDITIONS.

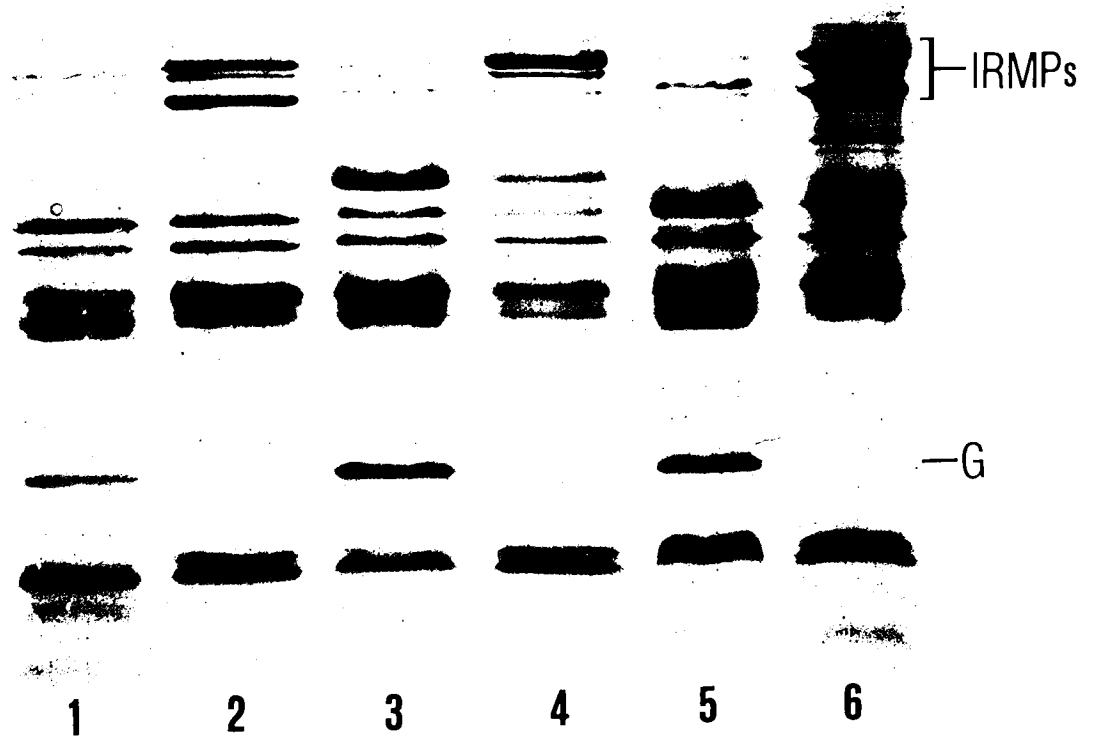
The protein profile of sarkosyl-extracted OMPs prepared from early stationary phase cells of *P. aeruginosa* strains PAO1, 6750 and PAJ grown in CDM+Fe or CDM-Fe are shown (Fig 4.3). The resulting profiles conformed to the basic pattern described by Mizuno and Kageyama (1978). Hancock and Carey (1979) have demonstrated the existence of between 6-9 major OMPs (depending on the growth conditions) and have developed a set of guidelines for electrophoretic analysis of these proteins. This, in conjunction with the new nomenclature proposed by Hancock *et al.*, (1990) was adopted in this study.

OprG expression was repressed by low iron concentrations and induced by high iron concentrations. The expression of OprG at this stage of growth was found to be a more sensitive indication of iron-stress than the presence of iron-regulated outer membrane proteins (IRMPs). *P. aeruginosa* IRMPs are difficult to suppress totally when the cells are grown in CDM+Fe, possibly because at physiological pH in an aerobic environment iron predominates as a highly insoluble ferric hydroxide polymer (Bullen, 1981). The induction of a low molecular weight 14 kDa IRMP (the receptor for ferripyochelin) under conditions of iron-depletion (Sokol and Woods, 1983; 1984; Sokol, 1984) was not detected during this study (see section 1.3.1.1)

4.4 EFFECT OF IRON CONCENTRATION UPON IRMP AND OprG EXPRESSION IN *P. aeruginosa*.

Figure 4.4.1 shows *P. aeruginosa* PAO1 cultivated to early stationary phase in CDM-Fe

Fig 4.3



Coomassie blue-stained SDS-PAGE showing the effects of iron on the expression of OprG in the OM of early stationary phase cells. Shown are *P. aeruginosa* strains 6750 (lanes 1 and 2), PAO1 (lanes 3 and 4) and PAJ (lanes 5 and 6), which were grown in CDM+Fe (lanes 1, 3, 5) and CDM-Fe (lanes 2, 4, 6).

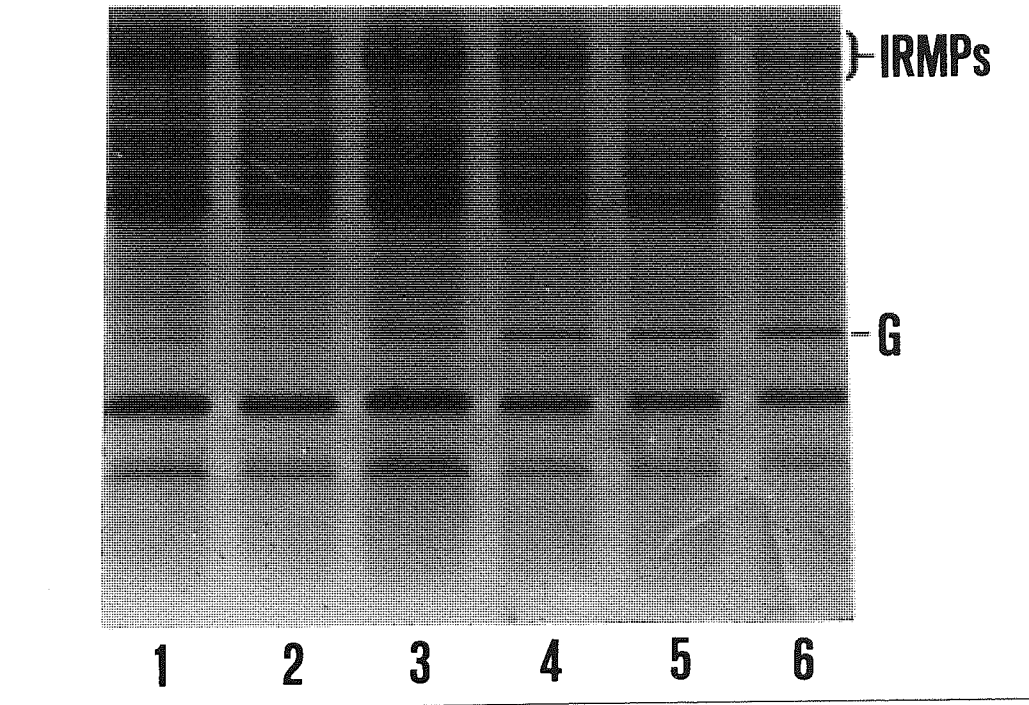
(lane 1) and with increasing concentrations of added iron; 0.1, 0.2, 0.3, 0.5 and 1 μ M (lanes 2, 3, 4, 5 and 6, respectively). The OMP profiles show a gradual induction of OprG with increasing media iron concentration and a parallel reduction in the expression of the IRMPs. SDS-PAGE and laser densitometry was used to quantify OprG as a percentage of the total OMPs (Fig 4.4.2). The amount of OprG incorporated into the OM of early stationary phase PAO1 cells was dependent upon media iron concentration, becoming fully expressed at 1 μ M iron. Concomitantly, the high molecular weight IRMPs responded in an opposite manner i.e. were fully expressed at very low iron concentrations and became gradually repressed as the iron concentration was raised.

4.5 ANALYSIS OF OprG EXPRESSION WITH PHASE OF GROWTH IN *P. aeruginosa* PAO1.

Batch cultures of *P. aeruginosa* PAO1 were grown in CDM+Fe or CDM-Fe and harvested at various points in the growth cycle and OMPs analysed by SDS-PAGE (Fig 4.5.1). Expression of OprG was determined at mid-log (4h), early-stationary (8h) and late-stationary phase (24h). The OMP profiles show OprG to be present throughout the growth cycle in iron-replete conditions and only present in late stationary phase cells when cultivated in iron-depleted media.

Expression of OprG in cells grown under iron-plentiful conditions varied throughout the growth cycle in batch culture, only becoming fully expressed at late stationary phase (Fig 4.5.2). Mid-log and early stationary phase cells grown in CDM-Fe possessed negligible amounts by comparison, although these cells began to accumulate OprG as they entered late stationary phase (16-24h). Since overnight cultures of bacteria are generally used in studies of cell physiology, and cultures are often in the stationary phase of growth, this can therefore give rise to cell populations that are totally unrepresentative of the environment outside the test-tube (*in vivo* or in a natural ecosystem) which is highly competitive and causes specialised adaptation for each circumstance (Gilbert *et al.*, 1987); this may explain why OprG sometimes appears in the OM of *P. aeruginosa* grown in CDM-Fe (Anwar *et al.*, 1985).

Fig 4.4.1



Coomassie blue-stained SDS-PAGE showing the effect of increasing concentrations of iron on the expression of OprG in the OM of early stationary phase cells. Shown are *P. aeruginosa* PAO1 under CDM-Fe (lane 1), and with 0.1, 0.2, 0.3, 0.5 and 1 μ M iron (lanes 2, 3, 4, 5, 6, respectively).

Fig 4.4.2

Effect of iron concentration upon OprG and IRMP expression in the OM of early stationary phase *P. aeruginosa* PAO1. SDS-PAGE, in conjunction with laser densitometry was used to quantify OprG as a percentage (%) of the total OMPs.

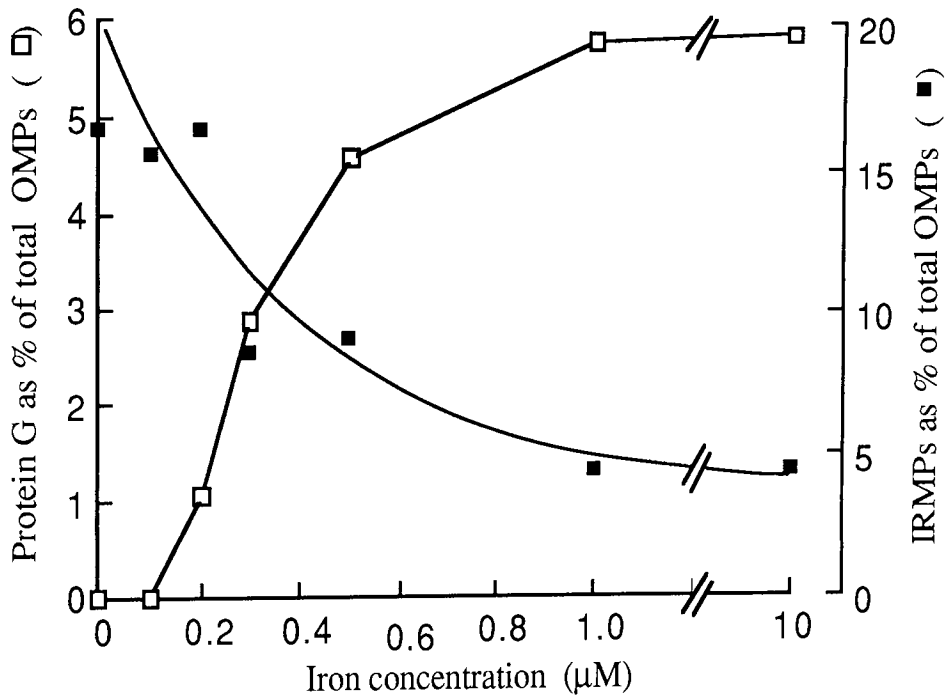
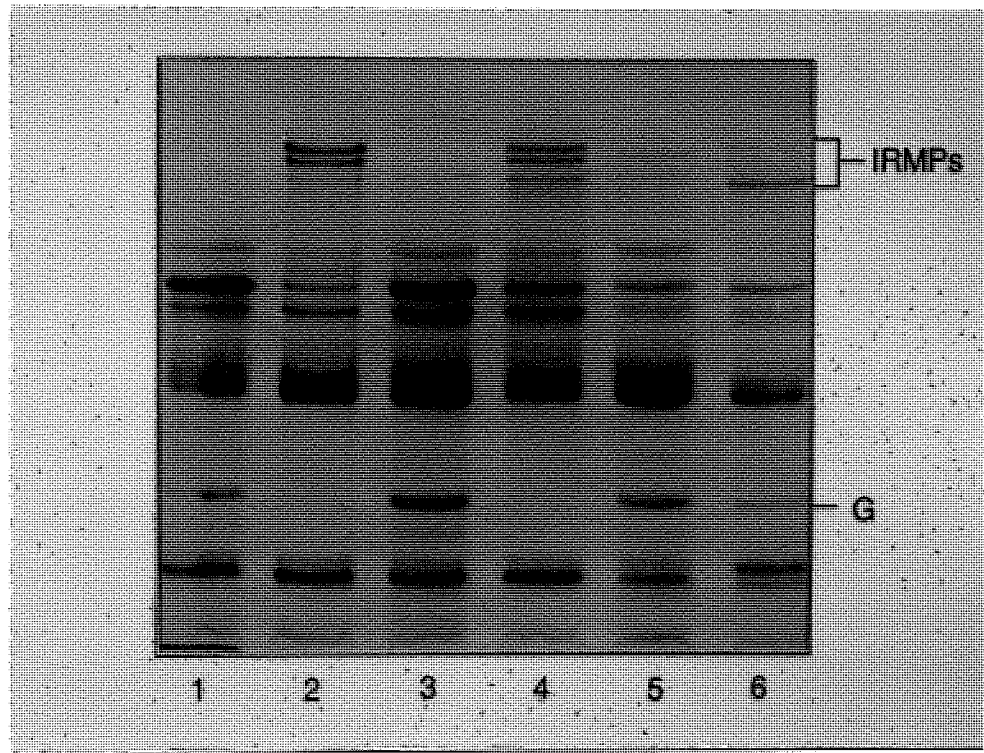


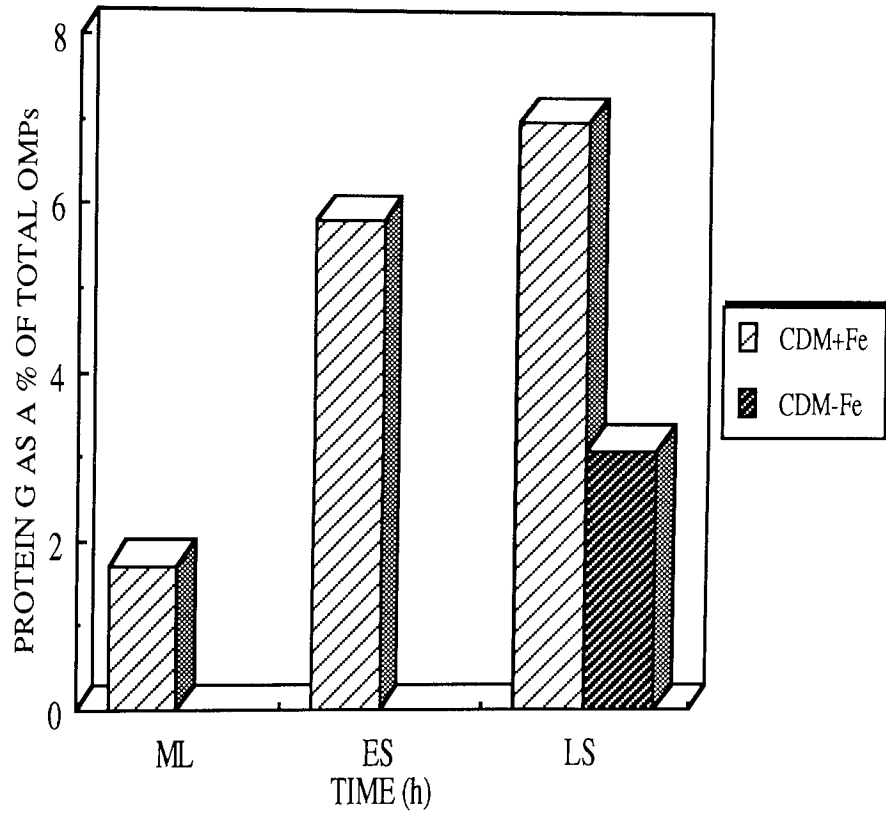
Fig 4.5.1



Coomassie blue-stained SDS-PAGE of OMP profiles of PAO1 grown in CDM+Fe (lanes 1, 3, 5) and CDM-Fe (lanes 2, 4, 6), harvested at mid-log (lanes 1 and 2), early stationary (lanes 3 and 4) and late stationary phase (lanes 5 and 6).

Fig 4.5.2

Batch culture of *P. aeruginosa* PAO1 grown in CDM+Fe or CDM-Fe and harvested at mid-log (ML), early stationary (ES) and late stationary (LS) phase. SDS-PAGE in conjunction with laser densitometry was used to quantify OprG as a percentage (%) of the total OMPs.



4.6 CULTIVATION OF *P. aeruginosa* IN IRON-PLENTIFUL AND IRON-DEPLETED MEDIA UNDER OXYGEN-DEFICIENT GROWTH CONDITIONS.

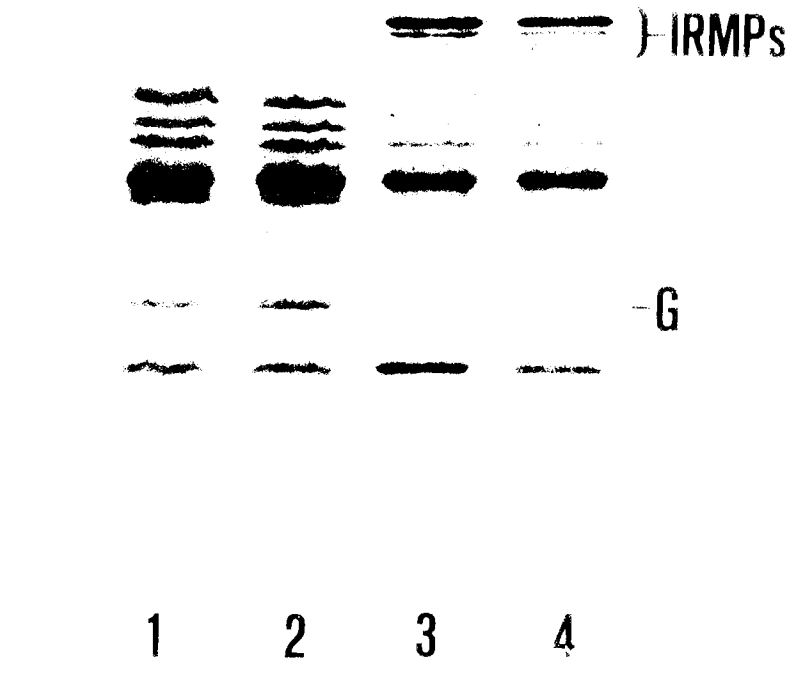
To determine whether OprG expression was regulated in response to general stress, *P. aeruginosa* PAO1 was grown in CDM+Fe or CDM-Fe to early stationary phase. In this study however, batch cultures in flasks containing both the normal (25%) and 50% of their volume of the appropriate CDM were used to observe the effect of oxygen-deficient growth conditions on the expression of OprG. This nutritional stress did not result in any noticeable changes in OprG expression (Fig 4.6). OprG was present only in cells cultivated in an iron-rich environment despite a reduction in the oxygen availability.

4.7 ANTIMICROBIAL BACTERICIDAL STUDIES IN BATCH CULTURE.

The plasticity of the bacterial envelope plays an important role in the survival of bacteria in hostile environments (Griffiths, 1983a; Brown and Williams, 1985b; Costerton, 1988). Variation in envelope characteristics, susceptibility to antibiotics and resistance to host defences in response to changes in the growth environment have been reported (Gilbert and Brown, 1978; Anwar *et al.*, 1983a; Brown and Williams, 1985a; Gilbert *et al.*, 1990; Evans *et al.*, 1990a, 1990b, 1991). Any experimental protocol designed to test the biological activities of antibiotics should take into account the effects of growth environment on the susceptibility of the test organism to those antibiotics. Consequently, it is now being realised that the physiological state of cells at the time of the test will greatly influence their outcome (Davis, 1974; Brown, 1977; Brown and Williams, 1985a; Gilbert *et al.*, 1987; Brown *et al.*, 1990).

The antimicrobial susceptibility studies (section 3.4.3) were used to assess the influence of growth phase and iron on the interaction of *P. aeruginosa* cells with two antimicrobial agents ciprofloxacin and gentamicin (see section 1.3.4), antibiotics of choice in the treatment of *Pseudomonas* infections (Barriere, 1986; Marcus and Goldman, 1986; Bow and Louie, 1989; Haron *et al.*, 1989; Maiche, 1991).

Fig 4.6



Batch cultures of PAO1 grown in CDM+Fe (lanes 1 and 2) or CDM-Fe (lanes 3 and 4), containing 25% (lanes 1 and 3) or 50% (lanes 2 and 4) of the flask volume.

4.7.1 Ciprofloxacin.

P. aeruginosa PAO1 grown under iron-replete or iron-deficient conditions in batch culture to mid-log (4h), early stationary (8h) and late stationary (24h), was subjected to the bactericidal assay using ciprofloxacin (Fig 4.7.1). The survival data of PAO1 cells grown in iron-plentiful media, show up to 10,000-fold greater sensitivity to ciprofloxacin compared to its iron-limited counterpart. The trend was influenced by growth phase, the cells being most sensitive in the exponential phase.

4.7.2 Gentamicin.

Figure 4.7.2 represents the survival data for gentamicin sensitivity of *P. aeruginosa* PAO1 cells grown under high- and low-iron conditions and at various points throughout the growth cycle. Sensitivity to gentamicin was up to 20-fold greater with high-iron-grown cells compared to those grown under conditions of low iron. As for ciprofloxacin, the presence of iron increases the sensitivity of this organism to gentamicin. In both cases susceptibility was influenced by growth phase, particularly for iron-replete cells.

4.7.3 Effects of physiological cation concentration on the bactericidal activity of ciprofloxacin.

The antagonism of quinolone antibacterials by multivalent metal ions, particularly magnesium is a recognised clinical interaction. Therapeutic failure due to a reduction in antibacterial potency can occur (Ratcliffe and Smith, 1983; Smith, 1989a, 1989b, 1990). The aim of this experiment was to investigate the influence of magnesium (Mg^{2+}) on the bactericidal activity of ciprofloxacin.

Susceptibility assays on cells grown in CDM+Fe or CDM-Fe were conducted in standard CDM salts (0.4mM Mg^{2+}), high Mg^{2+} CDM salts (4mM) and low Mg^{2+} CDM salts (0.02mM). The controls for each also being conducted using the appropriate CDM. Viable counts were performed at the start and end of the experiment without antibiotic. A

Fig 4.7.1
 Ciprofloxacin bactericidal assay for *P. aeruginosa* PAO1 grown in batch culture to mid-log (ML), early stationary (ES) and late stationary (LS) phase. The closed symbols represent CDM+Fe and the open symbols CDM-Fe cultures.

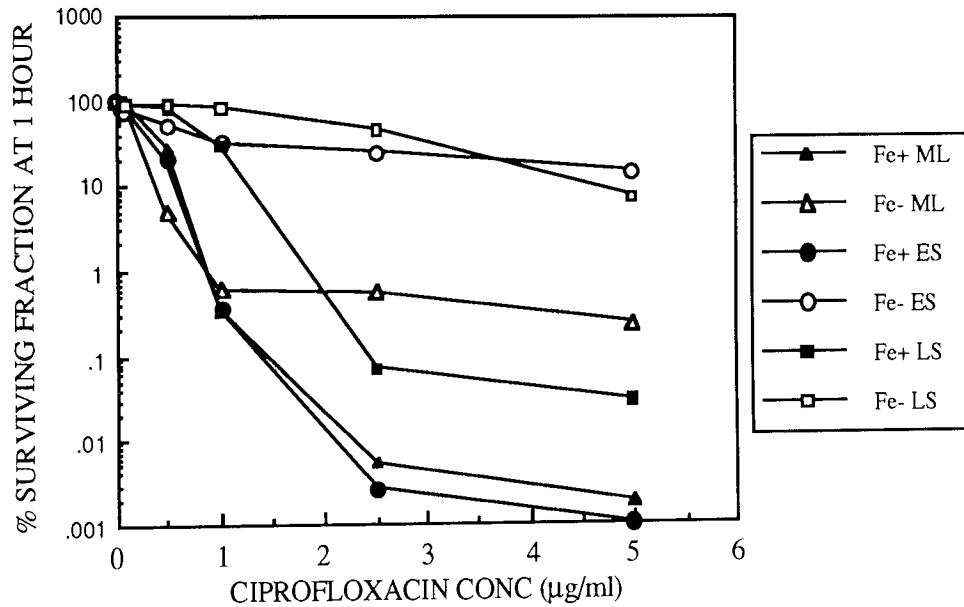
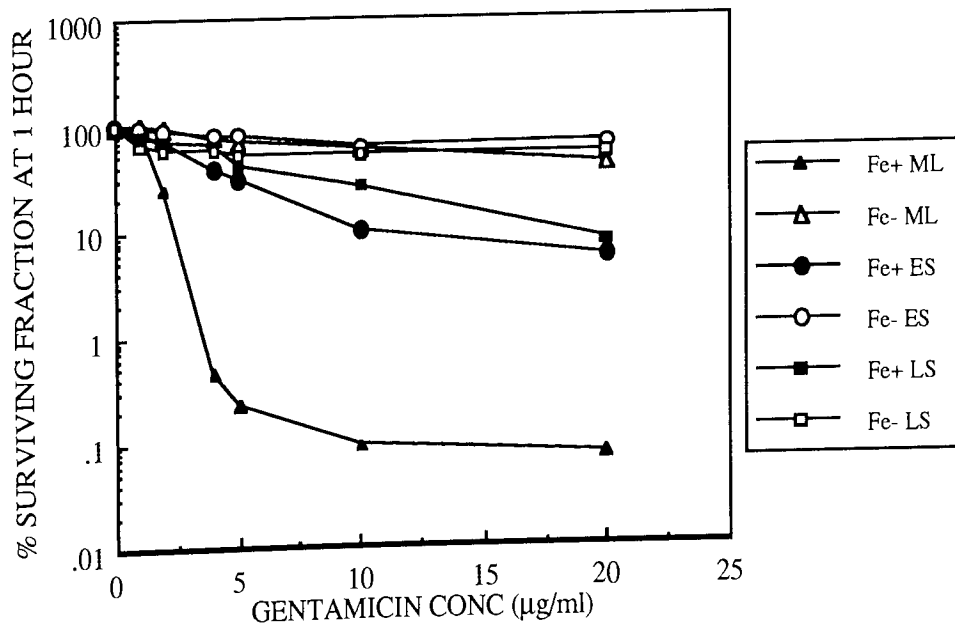


Fig 4.7.2
 Gentamicin bactericidal assay for *P. aeruginosa* PAO1 grown in batch culture to mid-log (ML), early stationary (ES) and late stationary (LS) phase. The closed symbols represent CDM+Fe and the open symbols CDM-Fe cultures.



ciprofloxacin concentration of $2.5\mu\text{g.ml}^{-1}$ was selected since previous experiments (section 4.7.1), demonstrated that this concentration gave a suitable level of killing at 1h. Table 4.7.3 shows that bactericidal susceptibilities were the same in the presence of 0.02mM and 0.4mM Mg^{2+} , but cells were 70-fold more resistant when assayed in the presence of 4mM Mg^{2+} .

Table 4.7.3 The effect of magnesium concentration on the bactericidal activity of ciprofloxacin.

Ciprofloxacin conc. ($\mu\text{g.ml}^{-1}$)	Magnesium conc. (mM)	% Surviving fraction
control	0.02	100
2.5	0.02	0.123

control	0.4	100
2.5	0.4	0.104

control	4.0	100
2.5	4.0	76.69

4.8 CHEMOSTAT STUDIES.

The closed environment of batch culture is gradually modified by cells until it no longer supports rapid growth. It is inherently difficult therefore, to study the influence of growth rate *per se* on cell physiology and associated properties in batch culture (Brown *et al.*, 1990). An open continuous culture can maintain cells growing under defined steady-state conditions (section 3.2.2). The chemostat allows specific growth rate (μ) to be controlled with minimal changes in the physicochemical environment of the cells. Growth rate control and application of particular nutrient deprivations in the chemostat have been used to model natural open growth systems such as infections.

The method of continuous culture was employed in this study to evaluate the effects of growth rate on OMP profiles of *P. aeruginosa*, particularly on OprG expression, and the subsequent susceptibility of these cells to the antimicrobial agents, ciprofloxacin and gentamicin.

4.8.1 SDS-PAGE of OMPs of *P. aeruginosa* grown in continuous culture.

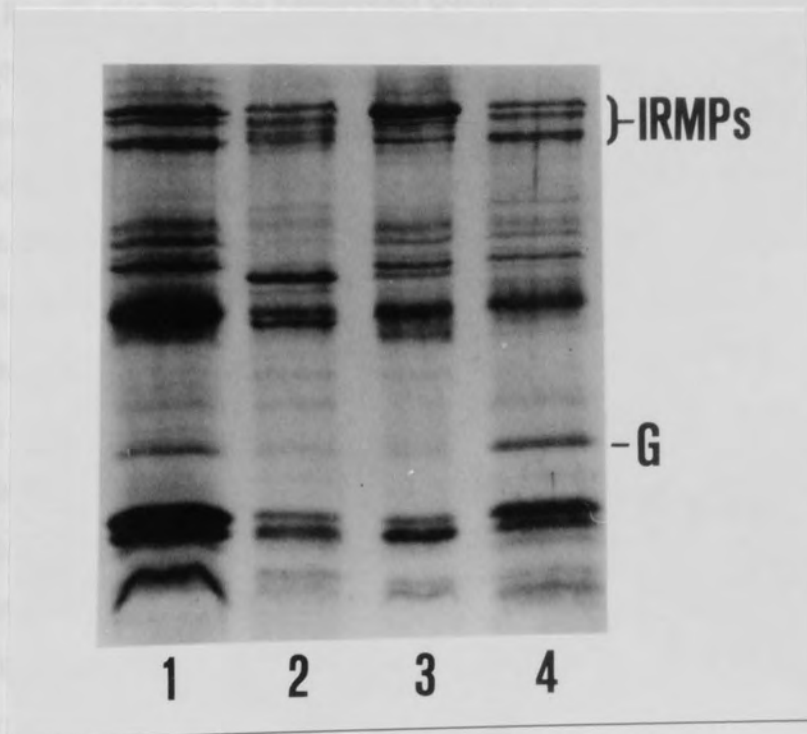
The OMP profiles of *P. aeruginosa* PAO1 grown in iron-limited (CDM-Fe) chemostat culture at slow ($D= 0.11\text{h}^{-1}$), medium ($D= 0.288\text{h}^{-1}$) and fast growth rates ($D= 0.44\text{h}^{-1}$) are shown (Fig 4.8.1). The IRMPs were fully expressed at all growth rates and OprG expression was shown to be growth-rate-dependent. Reduced growth rates ($D= 0.11\text{h}^{-1}$) led to an increase in OprG expression. This has been previously observed for *P. aeruginosa* M7 (Yates *et al.*, 1989), where increased doubling times led to increased amounts of OprG being expressed.

In preliminary experiments at a slow growth rate ($D= 0.11\text{h}^{-1}$), growth occurred as sessile biofilm populations adherent to the glassware (sinter for aeration) rather than as planktonic cells. These biofilm cells were removed by saline washes and harvested to prepare OMs. Figure 4.8.1, lane 4, shows the resultant OMP profile. Interestingly, OprG is strongly expressed in the adherent biofilm population of cells. The OMP profiles obtained from this biofilm resembles the profile of the iron-limited planktonic cells at the same growth rate ($D= 0.11\text{h}^{-1}$, lane 1) and that obtained from CDM-Fe late stationary phase batch culture (Fig 4.5.1, lane 6).

4.8.2 Antimicrobial bactericidal studies on chemostat cultures.

One of the most important factors in determining the outcome of an infection is the response of the organism to the antibiotic therapy. However, the *in vitro* bactericidal activity of an antibiotic does not always correlate with therapeutic efficacy. One explanation is the probable phenotypic differences between bacteria growing in the nutritionally limited *in vivo* environment and those growing under optimum conditions in the laboratory. A slow growth rate and a restricted availability of iron (Neilands, 1981b; Griffiths *et al.*, 1983;

Fig 4.8.1



Coomassie blue-stained SDS-PAGE OMP profiles of PAO1 grown in iron-limited chemostat culture at slow ($D = 0.11 \text{ h}^{-1}$, lane 1), medium ($D = 0.288 \text{ h}^{-1}$, lane 2), and fast growth rates ($D = 0.44 \text{ h}^{-1}$, lane 3). Lane 4 is the OMP profile of biofilm cells adhering to the glassware (sinter for aeration).

Anwar *et al.*, 1984; Brown *et al.*, 1984; Shand *et al.*, 1985; Ward *et al.*, 1988) and possibly of other nutrients appear to be characteristic of many infections *in vivo*. These parameters have been shown to be important environmental factors that govern the physiological state of the bacterial pathogens *in vivo* (Weinberg, 1978; Brown *et al.*, 1984; Weinberg, 1984; Brown and Williams, 1985b).

The effect of growth rate upon the bactericidal activity of antimicrobial agents in unattached planktonic populations was investigated using chemostat cultures. *P. aeruginosa* PAO1 grown in iron-limited (CDM-Fe) continuous culture at slow ($D=0.11\text{h}^{-1}$), medium ($D=0.288\text{h}^{-1}$) and fast growth rates ($D=0.44\text{h}^{-1}$) was studied. The survival data both for ciprofloxacin (Fig 4.8.2.1) and gentamicin (Fig 4.8.2.2) show a growth-rate-dependent effect with a significant increase in sensitivity in fast-growing cells. The maximum antibiotic concentration used ($5\mu\text{g}\cdot\text{ml}^{-1}$ and $20\mu\text{g}\cdot\text{ml}^{-1}$ for ciprofloxacin and gentamicin, respectively) was 5x MIC and therefore the results are comparable. Such concentrations are achievable *in vivo* (Campoli-Richards *et al.*, 1989), although not desirable for gentamicin due to problems of ototoxicity and nephrotoxicity (Bianco *et al.*, 1989). These concentrations were employed in the present study in order to differentiate between susceptibilities at various growth rates in the chemostat and at different phases of growth in batch experiments.

There was no sign of the paradoxical dose-response for *P. aeruginosa* reported by Crumplin and Smith, (1975) and Smith, (1986b) i.e. where the bactericidal activity increases with concentration until the optimal bactericidal concentration is reached. Higher concentrations become less bactericidal. Smith (1986b) reported that this occurred at concentrations of ciprofloxacin greater than 10x MIC, so perhaps if higher drug concentrations were used this effect would have been apparent. The absence of this biphasic effect has also been observed by other workers (Eliopoulos *et al.*, 1984; Wood, 1990; Evans *et al.*, 1991).

Fig 4.8.2.1
Ciprofloxacin bactericidal assay for *P. aeruginosa* PAO1 grown under iron-limitation at different dilution rates in the chemostat.

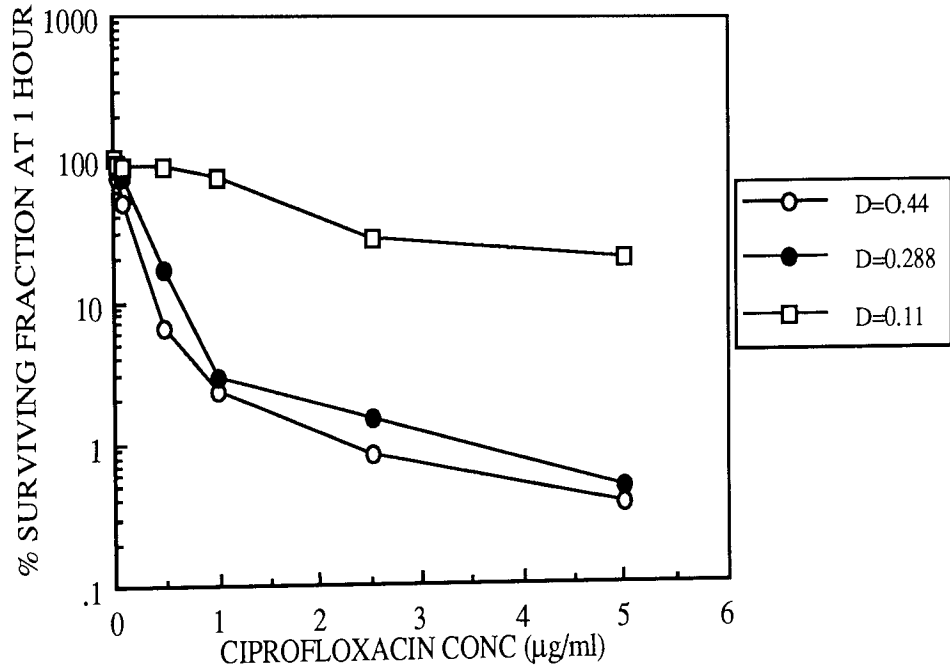
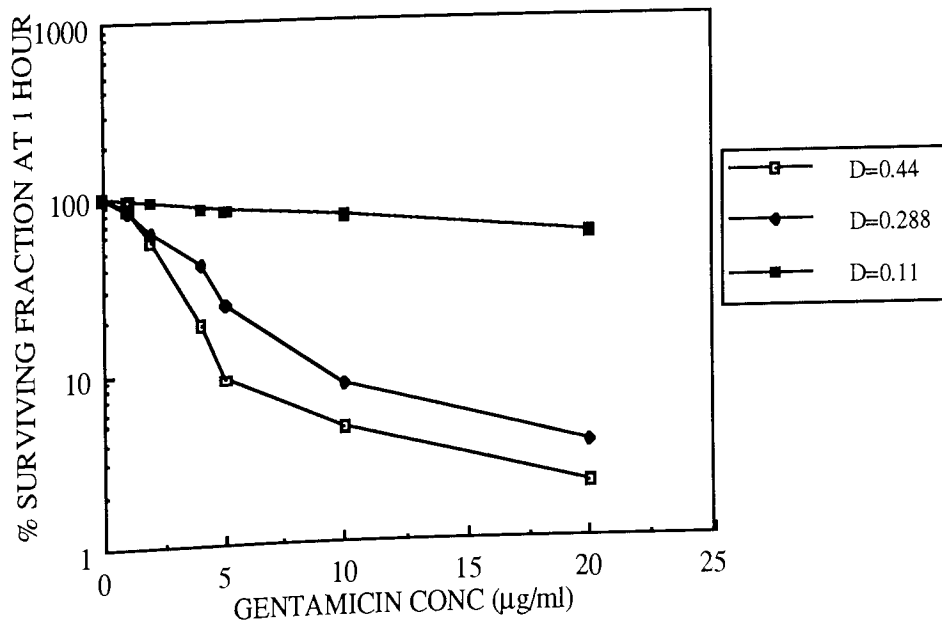


Fig 4.8.2.2
Gentamicin bactericidal assay for *P. aeruginosa* PAO1 grown under iron-limitation at different dilution rates in the chemostat.



4.9 SELECTION OF RESISTANCE IN *P. aeruginosa*.

Serial subculture of *P. aeruginosa* PAO1 in the presence of increasing incremental concentrations of ciprofloxacin resulted in the rapid selection of resistant cells (section 3.4.4). Figure 4.9 shows the development of resistance by change in MIC during serial passage through ciprofloxacin-containing CDM under both +Fe and -Fe conditions. There was no tendency for the acquired resistance to plateau at 16 x the initial MIC or after the tenth passage as observed by Reeves *et al.*, (1984a). The final MICs were 2, 10, 20 and 30-fold greater than the original MIC (1 μ g.ml⁻¹). Such a technique was employed in the present study to provide a quinolone-resistant variant for comparison of surface properties.

4.9.1 Measurement of bacterial growth of ciprofloxacin-resistant variants.

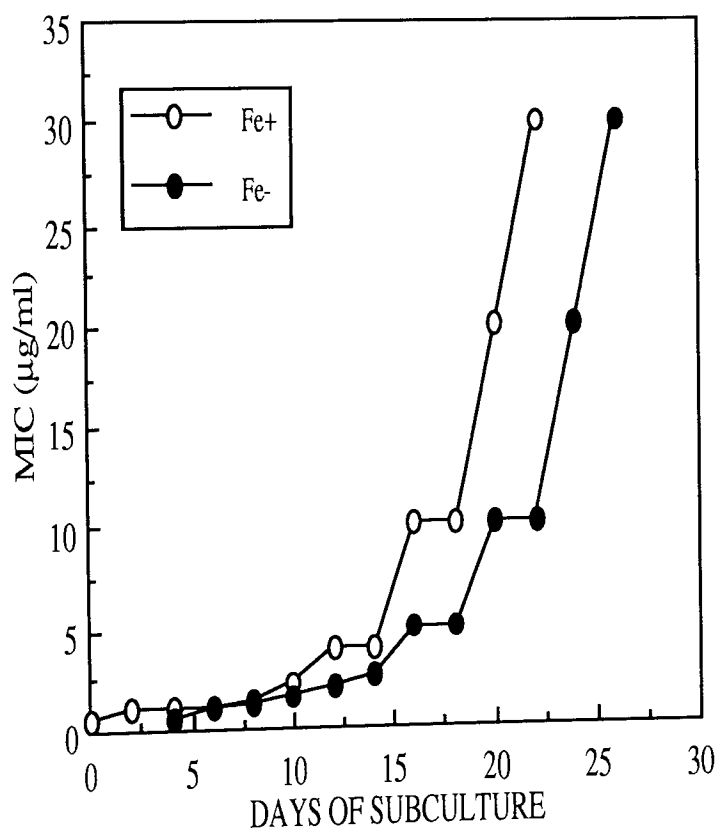
As the MIC increases, the ciprofloxacin-resistant variants grown in CDM+Fe (Fig 4.9.1.1) or CDM-Fe (Fig 4.9.1.2) show an increase in lag time, the doubling time and the final optical density compared to the original susceptible isolates (table 4.9.1).

Table 4.9.1 Growth of *P. aeruginosa* PAO1 and its ciprofloxacin-resistant variants.

Organism	Lag time (h)	Doubling time (min)	Final OD _{470nm}
CDM+Fe			
PAO1	1	60	4.05
PAO1- 2x MIC	4	120	3.05
PAO1- 10x MIC	5	126	2.04
PAO1- 20x MIC	5	126	1.57
PAO1- 30x MIC	6	138	1.35
CDM-Fe			
PAO1	2	60	0.95
PAO1- 2x MIC	4	78	0.79
PAO1- 10x MIC	5	132	0.67
PAO1- 20x MIC	5	138	0.615
PAO1- 30x MIC	6	138	0.52

Fig 4.9

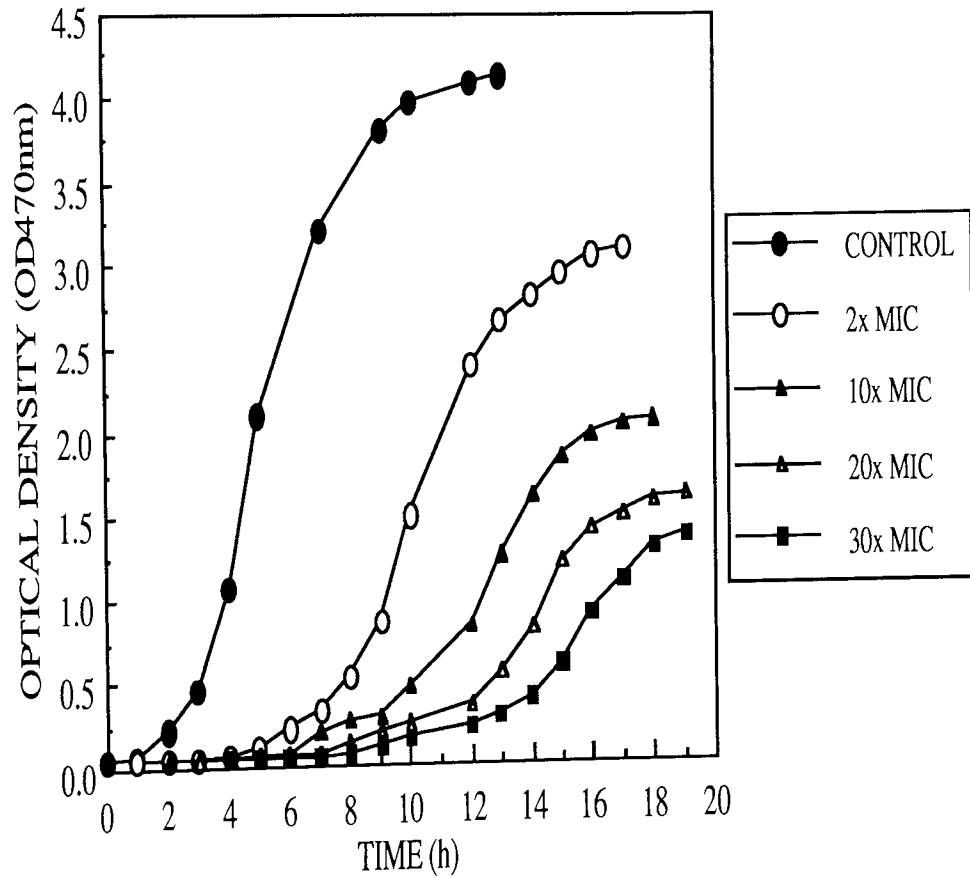
MIC for *P. aeruginosa* PAO1 exposed to repeated subculture in CDM containing ciprofloxacin under iron-plentiful and iron-depleted conditions.



The curve for CDM-Fe has been displaced by 4 days for clarity

Fig 4.9.1.1

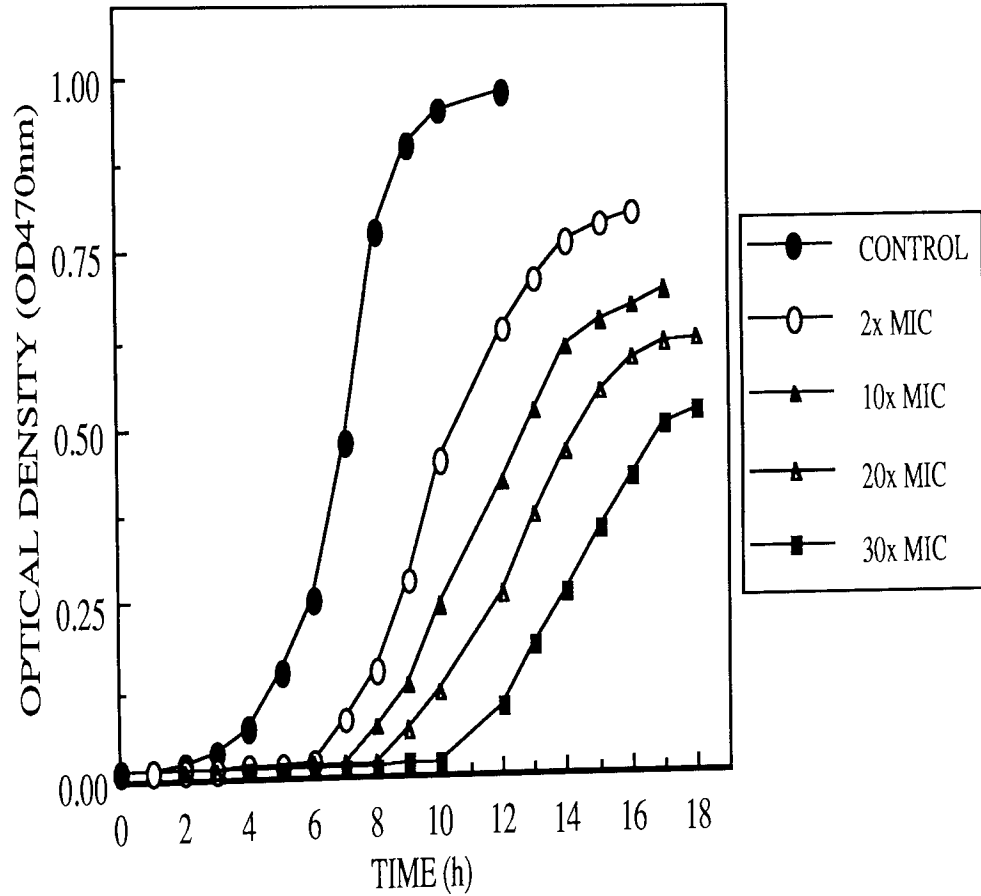
Growth of *P. aeruginosa* PAO1 and its ciprofloxacin-resistant variants at 2, 10, 20 and 30 x the original MIC, under iron-plentiful conditions.



The curves have been offset for clarity.

Fig 4.9.1.2

Growth of *P. aeruginosa* PAO1 and its ciprofloxacin-resistant variants at 2, 10, 20 and 30 x the original MIC, under iron-depleted conditions.



The curves have been offset for clarity.

A comparison is made of the surface properties of susceptible and resistant isolates. These were assessed by SDS-PAGE of bacterial OMPs, determination of the relative cell surface hydrophobicity evaluated by the bacterial adherence to hydrocarbon (BATH) assay, SDS-PAGE of LPS samples, KDO assay and phenol-sulphuric acid assay.

4.9.2 Analysis of OMP profiles under iron-plentiful and iron-depleted conditions.

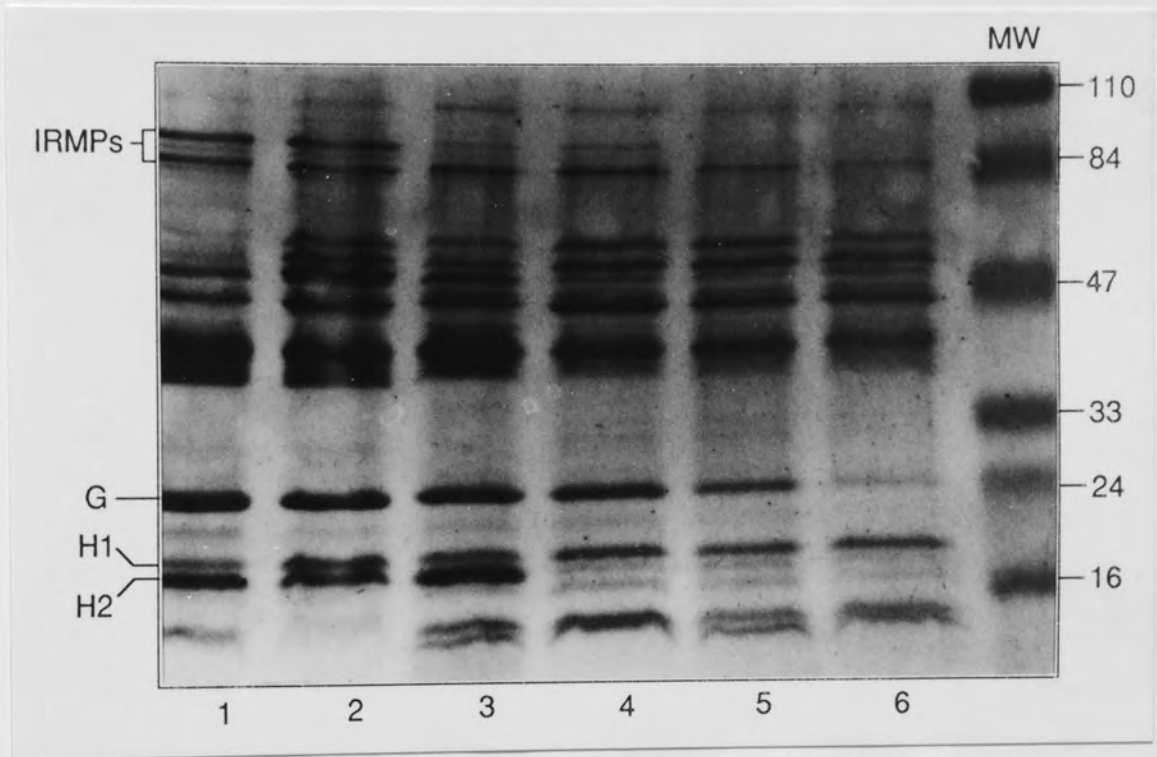
The coomassie blue-stained OMP profiles of PAO1 and its ciprofloxacin-resistant variants grown under CDM+Fe (Fig 4.9.2.1) or CDM-Fe (Fig 4.9.2.2) harvested at early stationary phase are shown.

Under iron-replete conditions (Fig 4.9.2.1) a dose-dependent loss of OprG was seen, together with a decrease in proteins D2 (OprD) and H2 (OprL). There was also a gradual repression of IRMPs despite being under Fe⁺ conditions (see section 4.3). These effects were quantified with laser densitometry (Fig 4.9.2.3). The scans were performed on different gels and an average value taken. A progressive loss of OprG and OprL can be observed, together with an increase in protein H1 (OprH).

Whereas under iron-depleted conditions the coomassie blue-stained gel shows the expected absence of expression of OprG (Fig 4.9.2.2). In contrast to iron-replete conditions, alterations in OMP profiles appear to be minor. There is a loss in OprD and again an alteration in the expression of IRMPs was noted. However, the dramatic loss of OprL under iron-rich conditions was not evident. Laser gel scans show an absence of OprG and a marginal increase in OprH (Fig 4.8.2.4). There is a marked decrease in OprL. It is interesting to note that under these conditions the ciprofloxacin-resistant clinical isolate from a CF patient showed increased expression of OprH and a decreased expression of OprL compared to the susceptible isolate PAO1.

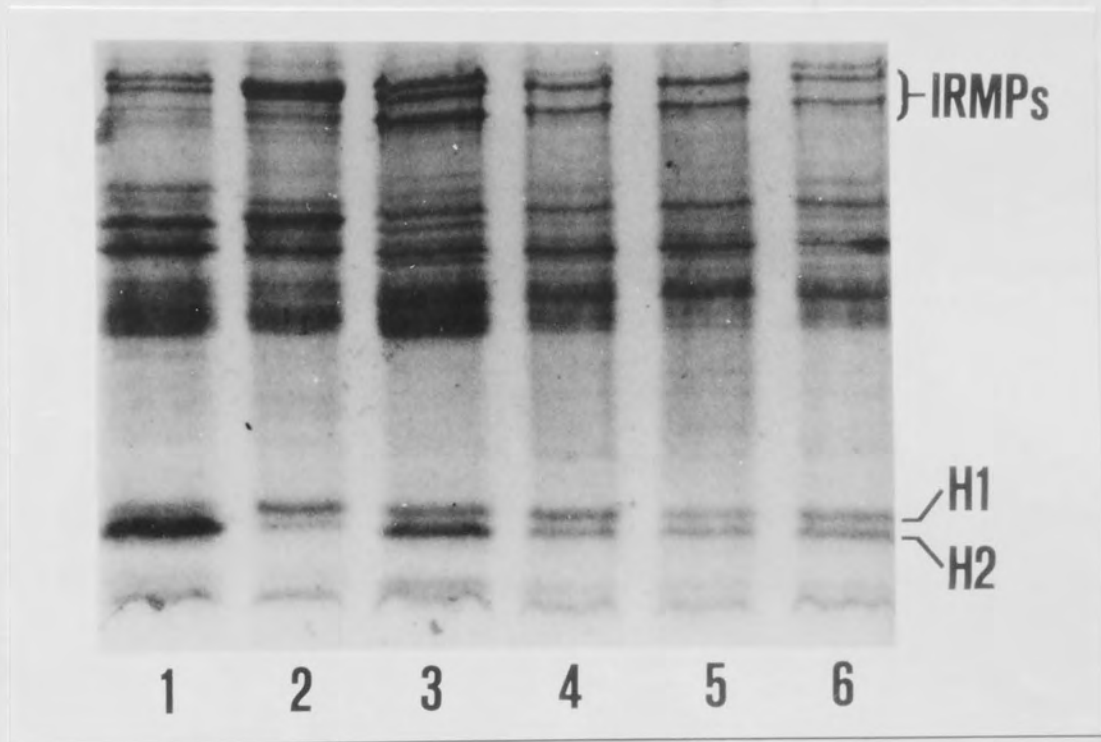
These highly resistant cells showed cross-resistance both to the 4-quinolone norfloxacin and the aminoglycoside gentamicin. The selection of resistance to quinolones in *P. aeruginosa* has also been associated with loss of sensitivity to nonquinolone classes of

Fig 4.9.2.1



Coomassie blue-stained OMP profiles of PAO1 grown in CDM+Fe (lane 1) and with $2\mu\text{g}\cdot\text{ml}^{-1}$ (lane 3), $10\mu\text{g}\cdot\text{ml}^{-1}$ (lane 4), $20\mu\text{g}\cdot\text{ml}^{-1}$ (lane 5), $30\mu\text{g}\cdot\text{ml}^{-1}$ ciprofloxacin (lane 6). Lane 2 is the OMP profile of a ciprofloxacin-resistant isolate from a CF patient. Molecular weight markers are shown in the right hand lane.

Fig 4.9.2.2



Coomassie blue-stained OMP profiles of PAO1 grown in CDM-Fe (lane 1) and with 2µg.ml⁻¹ (lane 3), 10µg.ml⁻¹ (lane 4), 20µg.ml⁻¹ (lane 5), 30µg.ml⁻¹ ciprofloxacin (lane 6). Lane 2 is the OMP profile of a ciprofloxacin-resistant isolate from a CF patient.

Fig 4.9.2.3
 Laser densitometry scans of Fig 4.9.2.1 showing the OMP profiles of *P. aeruginosa* PAO1 grown in CDM+Fe (lane 1) and with 2 $\mu\text{g.ml}^{-1}$ (lane 3), 10 $\mu\text{g.ml}^{-1}$ (lane 4), 20 $\mu\text{g.ml}^{-1}$ (lane 5), 30 $\mu\text{g.ml}^{-1}$ ciprofloxacin (lane 6). Lane 2 is the profile of a ciprofloxacin-resistant isolate from a CF patient.

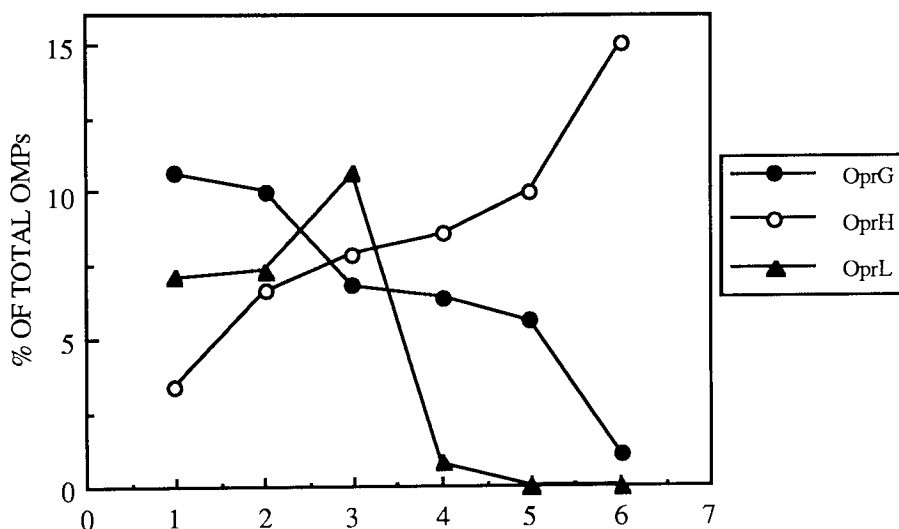
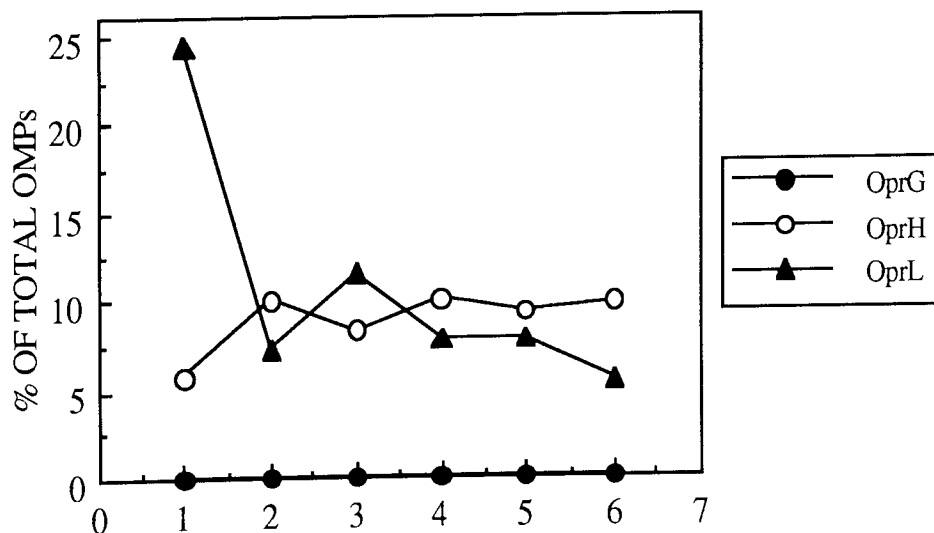


Fig 4.9.2.4
 Laser densitometry scans of Fig 4.9.2.2 showing the OMP profiles of *P. aeruginosa* PAO1 grown in CDM-Fe (lane 1) and with 2 $\mu\text{g.ml}^{-1}$ (lane 3), 10 $\mu\text{g.ml}^{-1}$ (lane 4), 20 $\mu\text{g.ml}^{-1}$ (lane 5), 30 $\mu\text{g.ml}^{-1}$ ciprofloxacin (lane 6). Lane 2 is the profile of a ciprofloxacin-resistant isolate from a CF patient.

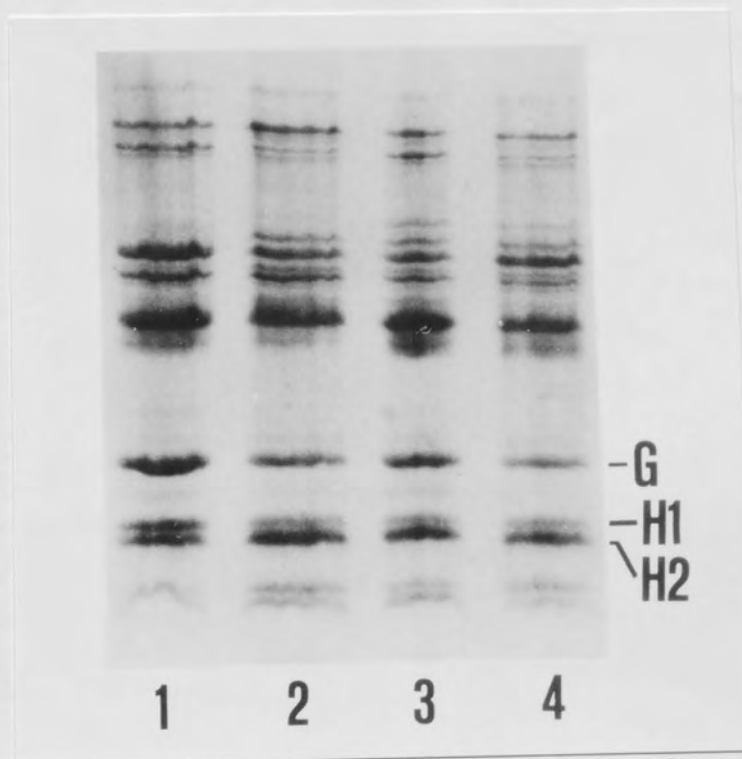


drugs, which suggests a decrease in drug permeation (Wolfson and Hooper, 1985). The stability of ciprofloxacin resistance in the PAO1 variants was tested in five serial passages of overnight cultures in drug-free medium. MICs were compared with those for the parenteral strains in simultaneous tests of drug susceptibility after each passage. After five serial passages the resistant isolates reverted to full sensitivity to ciprofloxacin, norfloxacin and gentamicin. Ecologic ascendancy of susceptible clones in the absence of a drug was assumed to reflect overgrowth by susceptible mutants, particularly since the growth rates of the susceptible and resistant strains were different. Repair of nonlethal drug-induced DNA defects is an alternative possibility. Although the MICs for the variants were found to be constant after one passage in antibiotic-free medium, the changes previously observed in the OMP profiles, reverted to those found in the original susceptible isolate, for CDM+Fe (Fig 4.9.2.5) and for CDM-Fe (Fig 4.9.2.6). On re-challenge of these ciprofloxacin-resistant isolates to the antibiotic the OMP profile remained the same for CDM+Fe (Fig 4.9.2.5) and CDM-Fe grown cells (Fig 4.9.2.6).

4.9.3 Cell surface hydrophobicity.

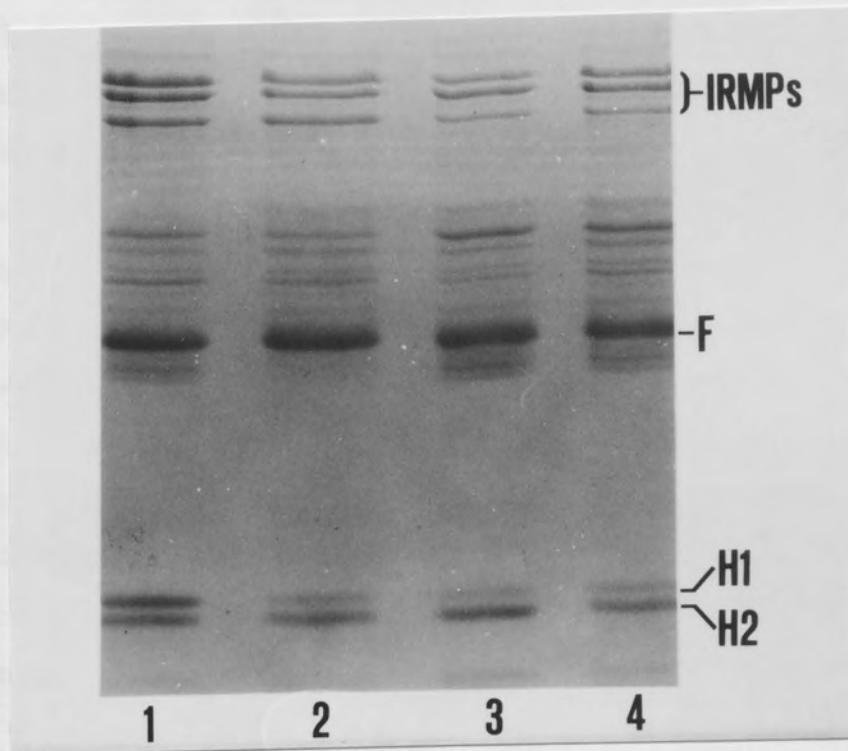
Antibiotics must penetrate the outer membrane to reach their target. The primary event, is therefore interaction of antimicrobial agents with the bacterial cell. This generally correlates with antimicrobial effect (Costerton and Cheng, 1975; Brown *et al.*, 1979). The hydrophobic nature of bacterial cells is important in the uptake of certain antibiotics (Nikaido and Vaara, 1985; Absolom, 1988). Moreover, bacterial hydrophobicity may affect adhesion to various surfaces. Adhesion to host surfaces such as teeth (Rosenberg and Kjelleberg, 1986) and surgical implants (Rosenberg and Kjelleberg, 1986) is recognised as one of the important initial steps in microbial pathogenesis. These early events involve non-specific, reversible interactions and bacterial hydrophobicity appears to be of major importance (Costerton, 1979; Vanhaecke and Pijck, 1988). There are many factors which can influence cell surface hydrophobicity in Gram-negative bacteria, including expression of various OMPs, LPS, phospholipids, associated divalent cations, particularly magnesium, and the presence or absence of an exopolysaccharide capsule (glycocalyx; Nikaido and Nakae, 1979; Nikaido and Vaara, 1985; Rosenberg and Kjelleberg, 1986; Costerton *et al.*, 1987).

Fig 4.9.2.5



Coomassie blue-stained OMP profiles of PAO1 grown in CDM+Fe (lane 1) and with $10\mu\text{g.ml}^{-1}$ (lane 2), $20\mu\text{g.ml}^{-1}$ (lane 3), $30\mu\text{g.ml}^{-1}$ ciprofloxacin (lane 4), after one passage in antibiotic-free media.

Fig 4.9.2.6



Coomassie blue-stained OMP profiles of PAO1 grown in CDM-Fe (lane 1) and with $10\mu\text{g.ml}^{-1}$ (lane 2), $20\mu\text{g.ml}^{-1}$ (lane 3), $30\mu\text{g.ml}^{-1}$ ciprofloxacin (lane 4), after one passage in antibiotic-free media.

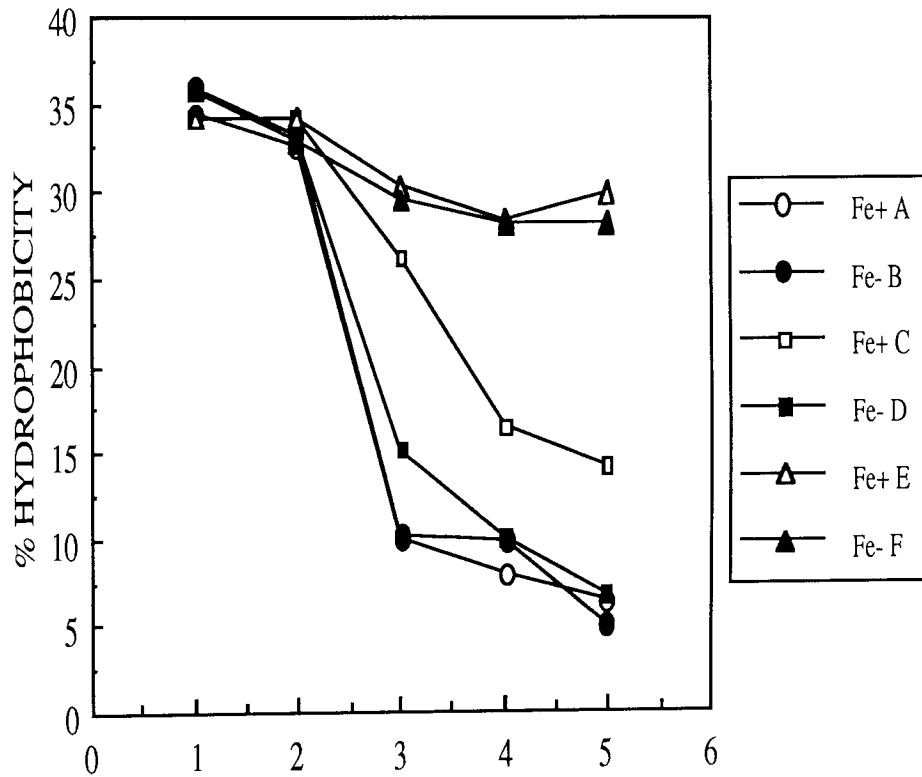
In the present study, bacterial adherence to hydrocarbon (BATH) has been utilised to evaluate changes in surface hydrophobicity of *P. aeruginosa* PAO1 cells selected to resist ciprofloxacin, cultivated under high- and low-iron conditions. Such measurements are a non-specific method of assessing general changes resulting from compositional and/or structural changes in several surface components (Absolom, 1988). The BATH assay provides a rapid and convenient method of estimating surface hydrophobicity and is not prone to dehydration effects associated with contact angle measurements. Measurements were carried out in triplicate upon cells collected on two separate occasions and the values averaged (see section 3.4.10). Figure 4.9.3 shows the results obtained. Hydrophobicity decreased under both iron-plentiful and iron-depleted conditions as the level of resistance increased up to 30-fold. Iron deprivation is known to decrease the surface hydrophobicity of *Neisseria gonorrhoea* (Magnusson *et al.*, 1979), however iron appears to have no effect in these experiments. Also shown is the cell surface hydrophobicity for the same ciprofloxacin-resistant cells under both high- and low-iron conditions after one passage and five passages in antibiotic-free medium. The graph shows a progressive return to the percentage hydrophobicity of the original susceptible isolate.

4.9.4 Analysis of LPS from *P. aeruginosa* PAO1 and its ciprofloxacin-resistant variants.

LPS is known to contribute to resistance to β -lactams (Godfrey *et al.*, 1984) and aminoglycosides (Bryan *et al.*, 1984; Shearer and Legakis, 1985). Therefore, the purpose of this study was a comparative analysis of the LPS profiles from *P. aeruginosa* strain PAO1, which was susceptible to ciprofloxacin and its *in vitro* derivatives which were selected to intermediate and high levels of resistance, together with a ciprofloxacin-resistant clinical isolate. Although extracted LPS can be resolved by SDS-PAGE and the carbohydrate component visualised by silver staining (Tsai and Frasch, 1982), characterisation of LPS is not restricted to purified fractions. Whole cells can be solubilised and proteins digested by incubation in a buffer containing the enzyme proteinase K prior to analysis by SDS-PAGE (Hitchcock and Brown, 1983). This technique has been applied to a range of Gram-negative organisms, including OM preparations of *P. aeruginosa* (Engels *et al.*, 1985). Methods of OM preparation based on insolubility in detergents work by

Fig 4.9.3

Relationship between hydrophobicity and *P. aeruginosa* PAO1 grown in CDM+Fe (open symbols) or CDM-Fe (closed symbols) in lane 1, with $2\mu\text{g.ml}^{-1}$ (lane 2), $10\mu\text{g.ml}^{-1}$ (lane 3), $20\mu\text{g.ml}^{-1}$ (lane 4), $30\mu\text{g.ml}^{-1}$ ciprofloxacin (lane 5) represented by A and B. Then after one passage (C and D), and five passages in antibiotic-free media (E and F).



selectively leaving or extracting protein-LPS complexes (Poxton *et al.*, 1985) and may therefore not be representative of the cell surface components. Subsequently whole cell digests of *P. aeruginosa* were used in this study.

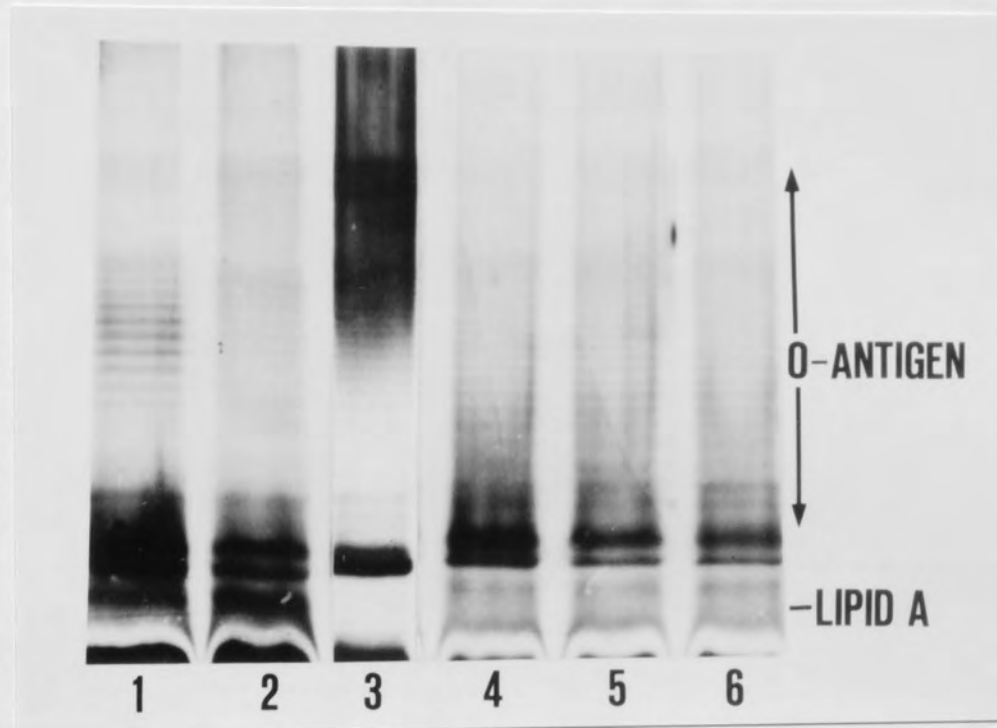
4.9.4.1 Characterisation of LPS by SDS-PAGE.

The SDS-PAGE silver-stained profile of proteinase K digests of whole cell lysates (section 3.4.13) grown under high- (Fig 4.9.4.1a) and low-iron (Fig 4.9.4.1b) conditions and harvested at early stationary phase are shown. Two distinct regions are shown, a fast-migrating low molecular weight region which represents the rough LPS (lipid A and core) and a ladderlike series of more slowly-migrating bands of LPS molecules possessing O-antigen structures with increasing numbers of repeating units, so decreasing their migration rate (Goldman and Leive, 1980; Palva and Makela, 1980), indicating a considerable degree of heterogeneity in the size of individual LPS molecules. All the isolates demonstrated similar profiles, there was no obvious deficiency in LPS synthesis for PAO1 or its resistant variants with the exception of the clinical isolate, which especially under iron-depletion appeared to express almost only core LPS (Fig 4.9.4.1b, lane 2). Double and triple loadings of the samples were also performed to ensure that any differences observed were not due to a loading effect (data not shown).

4.9.4.2 2-Keto 3-deoxy-D-manno-2-octulosonic acid (KDO) content of *P. aeruginosa* PAO1 and its ciprofloxacin-resistant variants.

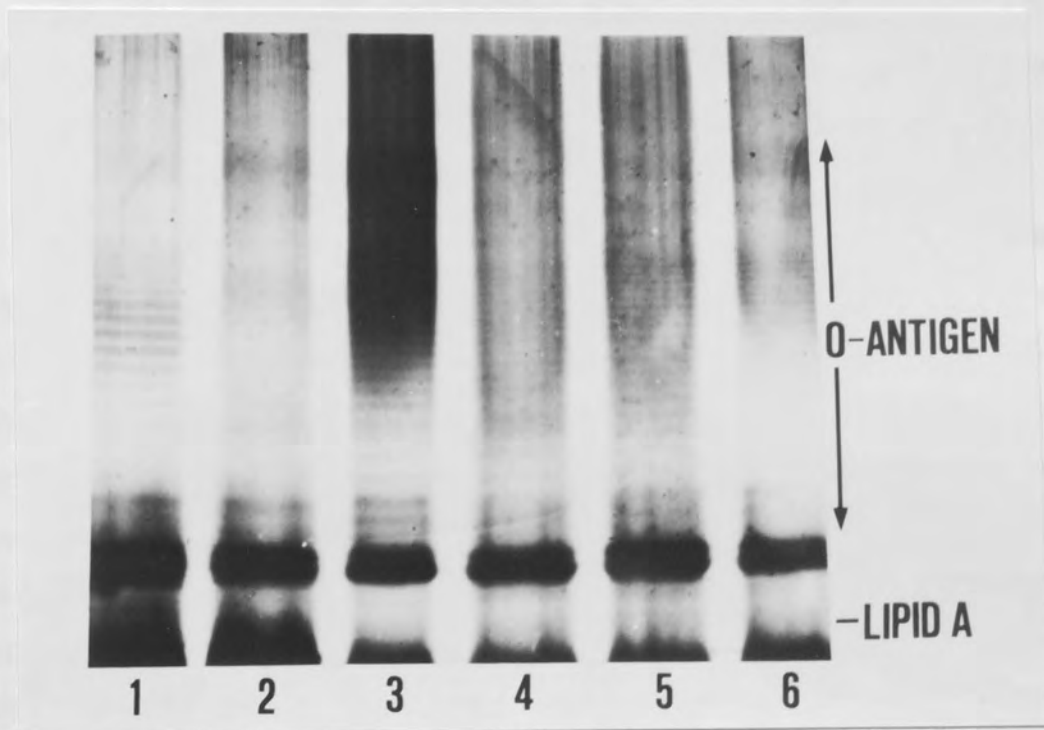
LPS has been shown to play an important role in resistance of many Gram-negative bacteria to various antibiotics (Conrad and Galanos, 1989). Consequently it was decided to investigate the LPS of *P. aeruginosa* PAO1 and its ciprofloxacin-resistant variants further in order to quantify any differences not visible by the LPS-PAGE technique. KDO has been used to estimate *P. aeruginosa* LPS (Gilbert and Brown, 1978; Miguez *et al.*, 1986) as the number of KDO units per molecule of LPS is relatively constant. Disadvantages associated with this technique include its non-specificity and the likelihood of interference by other bacterial cell components (see section 3.4.8).

Fig 4.9.4.1a



Silver-stained SDS-PAGE profiles of proteinase K digests of whole cell lysates of *P. aeruginosa* PAO1 grown in CDM+Fe (lane 1), and with 2µg.ml⁻¹ (lane 3), 10µg.ml⁻¹ (lane 4), 20µg.ml⁻¹ (lane 5), 30µg.ml⁻¹ ciprofloxacin (lane 6). Lane 2 is the profile of a ciprofloxacin-resistant isolate from a CF patient.

Fig 4.9.4.1b



Silver-stained SDS-PAGE profiles of proteinase K digests of whole cell lysates of *P. aeruginosa* PAO1 grown in CDM-Fe (lane 1), and with $2\mu\text{g.ml}^{-1}$ (lane 3), $10\mu\text{g.ml}^{-1}$ (lane 4), $20\mu\text{g.ml}^{-1}$ (lane 5), $30\mu\text{g.ml}^{-1}$ ciprofloxacin (lane 6). Lane 2 is the profile of a ciprofloxacin-resistant isolate from a CF patient.

The results of the KDO content for cells cultivated under high (Fig 4.9.4.2a) and low iron (Fig 4.9.4.2b) are shown. Each sample was standardised by obtaining a ratio to the protein content (μg of KDO per mg of protein). As the MIC for the ciprofloxacin-resistant strains increases up to 30-fold compared to the susceptible isolate, the KDO: protein ratio in the OM of both CDM+Fe and CDM-Fe grown cells decreased 5-fold indicating perturbed expression of LPS.

4.9.4.3 *Phenol-sulphuric acid assay to evaluate the total hexose content of P. aeruginosa PAO1 and its ciprofloxacin-resistant variants.*

The aim of this study was to gain further information regarding the LPS alterations by analysis of their chemical composition (section 3.4.9) in the ciprofloxacin sensitive and resistant isolates. A qualitative hexose analysis was conducted using the neutral sugar rhamnose as the standard since it is assumed to be a major constituent of the O-side chain (Conrad and Galanos, 1989). Also, its presence has been found to be conserved amongst *P. aeruginosa* strains (Kropinski *et al.*, 1982). The hexose content of cells grown under high (4.9.4.3a) and low iron (Fig 4.9.4.3b) are shown. Values were the mean of three determinations per sample and these were standardised by obtaining a ratio to the protein content (μg of rhamnose per mg of protein). For samples cultivated in high or low iron conditions, there was a marginal decrease compared to the value obtained for the original susceptible isolate PAO1. However, this difference was not considered to be significant. Conrad and Galanos (1989) found the concentration of rhamnose in an *in-vitro* polymyxin B-resistant isolate to be increased compared to those in PAO1. It is suggested that a loss of O-side chain (smooth to rough form) would result in an apparent relative increase of rhamnose. However, examination of the smooth profiles depicted by SDS-PAGE LPS analysis shows the profiles to be unaltered. It is probable, as suggested by Said *et al.*, (1987) that not all surface alterations have a direct bearing on resistance. The correlation of specific alterations and resistance is unclear and the molecular basis for adaptive resistance remains mechanistically unresolved. It is unlikely that the development of adaptive resistance to ciprofloxacin is the sole responsibility of a single component of the cell envelope such as LPS.

Fig 4.9.4.2a

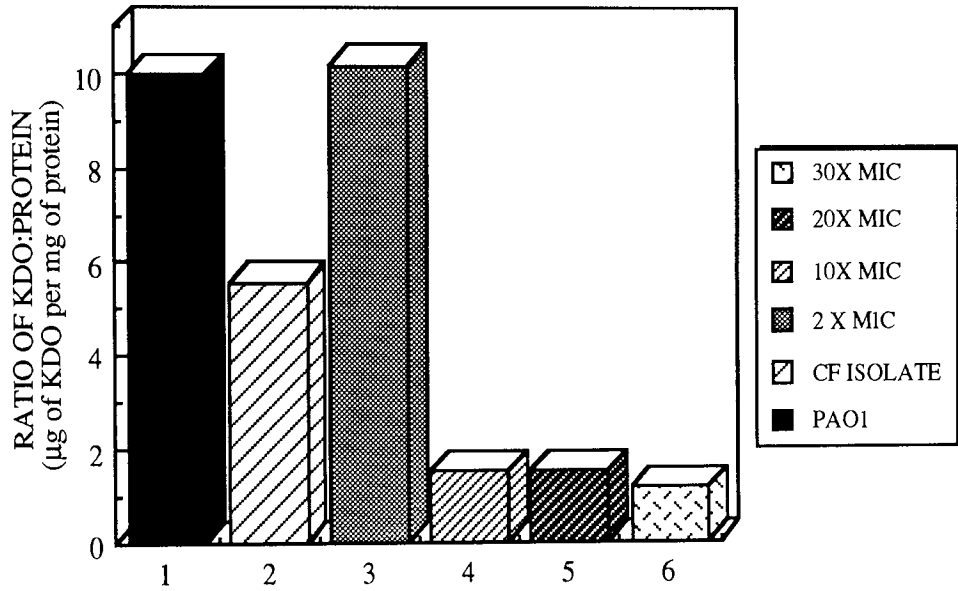
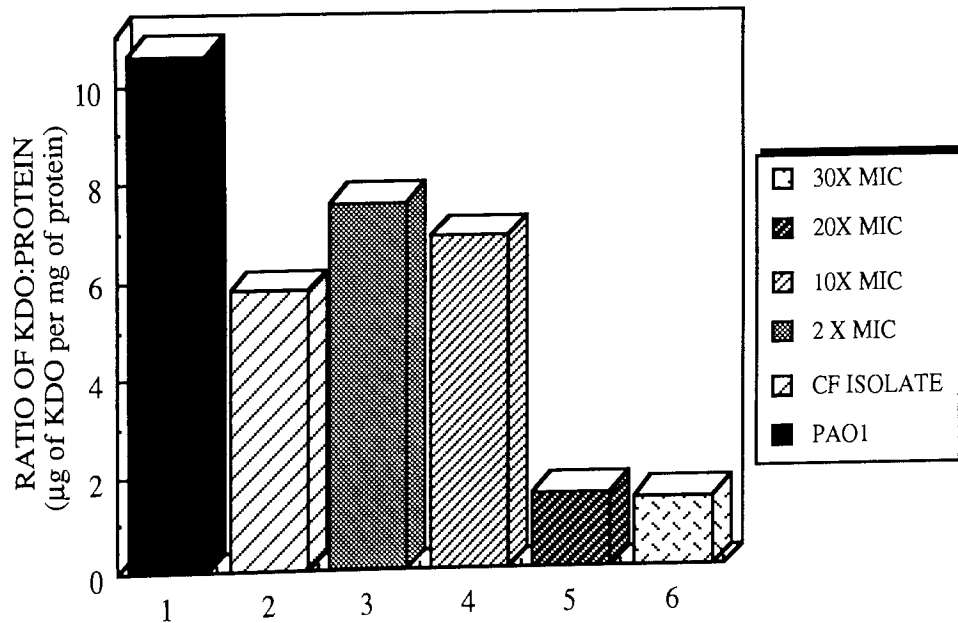


Fig 4.9.4.2b



The KDO content of LPS from *P. aeruginosa* PAO1 and its ciprofloxacin-resistant variants. Shown are CDM+Fe (Fig 4.9.4.2a) and CDM-Fe (Fig 4.9.4.2b) in lane 1, with $2\mu\text{g}\cdot\text{ml}^{-1}$, (lane 3), $10\mu\text{g}\cdot\text{ml}^{-1}$ (lane 4), $20\mu\text{g}\cdot\text{ml}^{-1}$ (lane 5), $30\mu\text{g}\cdot\text{ml}^{-1}$ ciprofloxacin (lane 6). Lane 2 represents a ciprofloxacin-resistant isolate from a CF patient.

Fig 4.9.4.3a

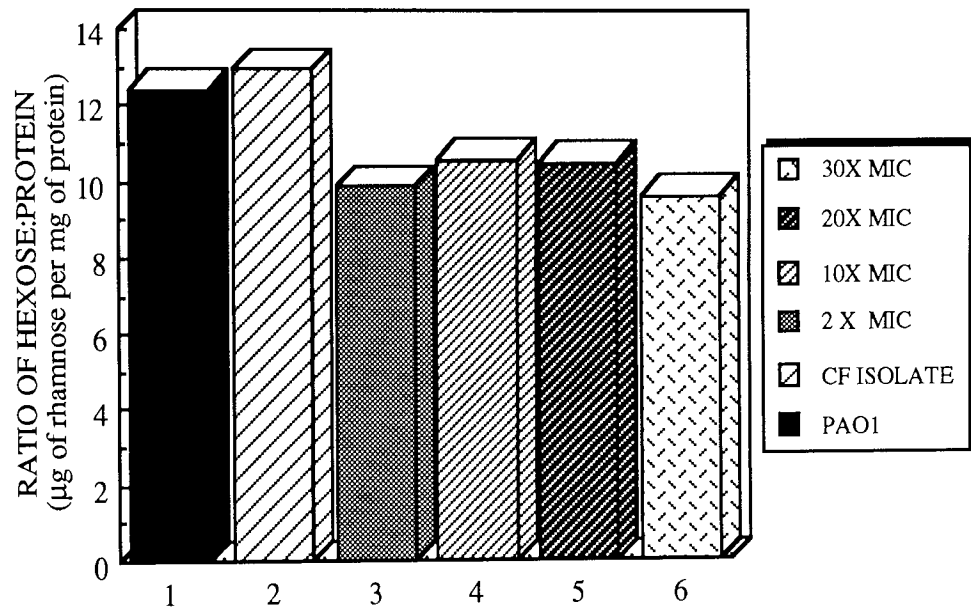
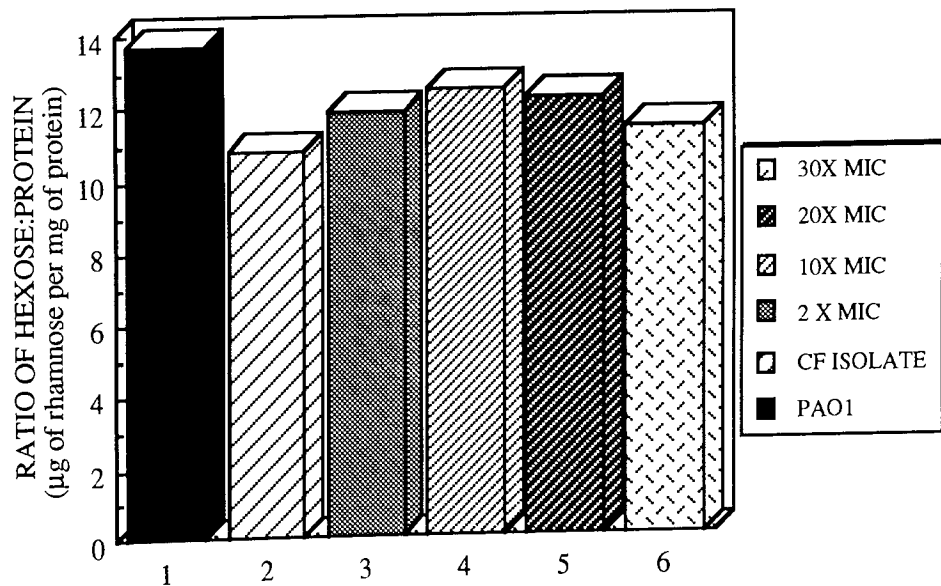


Fig 4.9.4.3b



The hexose content of LPS from *P. aeruginosa* PAO1 and its ciprofloxacin-resistant variants. Shown are CDM+Fe (Fig 4.9.4.3a) and CDM-Fe (Fig 4.9.4.3b) in lane 1, with $2\mu\text{g}\cdot\text{ml}^{-1}$, (lane 3), $10\mu\text{g}\cdot\text{ml}^{-1}$ (lane 4), $20\mu\text{g}\cdot\text{ml}^{-1}$ (lane 5), $30\mu\text{g}\cdot\text{ml}^{-1}$ ciprofloxacin (lane 6). Lane 2 represents a ciprofloxacin-resistant isolate from a CF patient.

4.10 DISCUSSION.

In all *P. aeruginosa* strains tested, including a clinical isolate, OprG expression was repressed by low iron and induced by high iron concentrations in the media. Suppression of OprG expression under iron limitation was also observed by Ohkawa *et al.*, (1980), Cochrane (1987), and Ward (1987), although they did not characterise this phenomenon further.

The amount of OprG incorporated into the OM of early stationary phase PAO1 was found to be dependent upon media iron concentration. Concomitantly, the IRMPs responded in an opposite manner, being fully expressed at very low iron concentrations and gradually repressed as the media iron concentration was raised. McIntosh and Earhart (1977) showed the IRMPs of *E. coli*, which serve as siderophore receptors, to behave in a similar manner. Such a result is to be expected for proteins associated with high-affinity uptake systems. It is conceivable that OprG comprises part of a low-affinity iron-uptake system which is repressed and superseded by the high-affinity system when iron levels are reduced. In *E. coli*, a membrane protein of molecular weight 90 kDa has been described to respond in a similar manner as OprG with regard media iron concentration (Klebba *et al.*, 1982). It has been suggested that this protein may serve a role in iron storage when cells are grown under iron-rich conditions (Klebba *et al.*, 1982).

Analysis of OMP profiles showed expression of OprG in both batch and chemostat cultures to be dependent both on the level of available iron and growth rate/phase. The expression of OprG noted with slow growth rate in the iron-limited chemostat, but not at higher growth rates, could be either a direct influence of growth rate itself or a secondary phenomenon due to growth rate affecting the iron status of the bacteria. Growth rate has previously been shown to affect the high-affinity iron-uptake system of *Klebsiella pneumoniae* (Lodge *et al.*, 1986). The results of these experiments suggest that the acquisition of iron is of relatively less importance in slow-growing *K. pneumoniae* than in those growing at near maximal rates, even in iron-limited cultures. This conclusion was supported by the observation that the high-affinity iron-uptake system was not fully expressed at the slowest growth rate in iron-limited or carbon-limited low iron chemostats.

The adherent (to the sinter) sessile biofilm population obtained during preliminary experiments at slow-growth rates in the chemostat produced an OMP profile resembling that from iron-limited planktonic cells at the slow-growth rate ($D= 0.11h^{-1}$) and that obtained from iron-deficient late stationary phase batch culture, particularly with respect to OprG expression. However, care should be taken in interpreting these results since this technique lacks effective growth rate control and therefore does not differentiate between properties attributable to growth rate and those associated with adhesion (Brown *et al.*, 1988a). However, growth rates in biofilms are generally thought to be slow and resembling those of stationary phase batch cultures (Brown *et al.*, 1988a). Many chronic infections, particularly those involving medical implants and prosthetic devices, involve bacterial consortia which grow slowly, under iron-limitation (Anwar *et al.*, 1984) as adherent biofilms within extended polysaccharide glycocalices (Brown and Williams, 1985b; Costerton *et al.*, 1987). *P. aeruginosa* CF lung isolate probed with the patients own serum has been found to recognise OprG (Anwar *et al.*, 1984; Dr. J. West, personal communication). Consequently OprG expression may be an important response to the environment encountered by bacteria *in vivo*.

The antimicrobial susceptibility studies conducted in batch culture showed that sensitivity to ciprofloxacin and gentamicin correlated with media iron concentration and the phase of growth.

Chemostat populations were extremely resistant both to ciprofloxacin and gentamicin at slow rates of growth, but displayed a significant progressive increase in susceptibility as growth rate was increased. This growth-rate-dependence of antibiotic susceptibility for planktonic (chemostat) cells is similar to observations made by other workers, with various antimicrobial agents on different organisms and under different nutritional stresses (Finch and Brown, 1975; Gilbert and Brown, 1980; Brown and Williams, 1985b; Cozens *et al.*, 1986; Evans *et al.*, 1990a; 1991).

There are few data presented on the effect of cultural conditions on OprG expression. Hancock and Carey (1979) have shown that it is a heat modifiable protein and that it did not serve as a receptor for any phage or pyocins with which they challenged their strains.

Whilst investigating the properties of *P. aeruginosa* OMP H1 (OprH), Nicas and Hancock (1980) noticed OprG to be influenced by the magnesium concentration of the medium. Magnesium depletion led to decreased OprG expression. However, the effects were minor when compared with those induced by media iron concentration described here. In contrast, when *P. aeruginosa* was subjected to magnesium- (Yates *et al.*, 1989) or oxygen-deficient growth conditions, OprG expression was not altered. These data suggest that its regulation is iron-specific and not responsive to non-specific environmental stress. These initial results support the hypothesis that OprG may comprise part of a low-affinity iron-uptake system, although, functions related to uptake of other nutrients cannot be ruled out.

Infection by *P. aeruginosa* is a serious problem, particularly in compromised hosts, because of its high levels of resistance to many antibacterial agents. Resistance to the fluoroquinolones in Gram-negative bacteria, including *P. aeruginosa* has been associated with modification of their intracellular target, DNA gyrase (Gellert *et al.*, 1977; Sato *et al.*, 1986; Inoue *et al.*, 1987) or alteration of outer membrane permeability (Wolfson and Hooper, 1985; Hirai *et al.*, 1986b; Hooper *et al.*, 1986; Robillard and Scarpa, 1988).

When cells were trained to resist up to 30x MIC of ciprofloxacin, they exhibited a longer lag phase before growth, a slower generation time and a lower final OD compared to the original susceptible isolate. Chamberland *et al.*, (1990) reported a similar effect for *in vivo* isolated ciprofloxacin-resistant cells and suggested that modification of the OM components such as OMPs or LPS may have had an effect on the capacity of these organisms to sustain growth.

Selection for quinolone resistance was associated with resistance to other antimicrobial agents, such as norfloxacin and gentamicin. Pleiotropic resistance to structurally unrelated drugs, together with the slower growth rate of these resistant isolates, indicates decreased drug permeation (Rella and Haas, 1982; Sanders *et al.*, 1984; Neu, 1988; Wolfson and Hooper, 1989). It has been suggested that the alteration of cell permeability was likely to result from modification of porin channels (Chamberland *et al.*, 1989; Wolfson *et al.*, 1989). This phenomenon has been studied in greatest detail in *E. coli*. Quinolones may penetrate the OM of *E. coli* via several different porins. OmpF is the preferred route of

entry, although OmpC and *phoE* maybe used when OmpF is absent (Chapman and Georgopapadakou, 1988; Mortimer *et al.*, 1991; Piddock, 1991). This has been demonstrated both by the use of defined porin-deficient strains (Hirai *et al.*, 1986a) and by the isolation of quinolone-resistant mutants that lack porins (Hirai *et al.*, 1986b; Hooper *et al.*, 1986). The accumulation of quinolones in mutants lacking all the major porins is reduced. However, as approximately 25% still enters the bacterial cell, the passage of quinolones through the OM is not limited to porins (Mortimer *et al.*, 1991).

There are several porin candidates in *P. aeruginosa*. The function of protein F (OprF) as a porin is currently the source of controversy. It has been suggested that OprF forms a relatively large pore (Hancock *et al.*, 1979; Yoshimura *et al.*, 1983; Woodruff and Hancock, 1988; Nikaido *et al.*, 1991) and that the unusual low permeability of *P. aeruginosa* results from the fact that more than 99% of OprF molecules are nonfunctional (Angus *et al.*, 1982; Nicas and Hancock, 1983b; Woodruff *et al.*, 1986). In contrast the small size of porin pores in *P. aeruginosa* has been suggested as the cause of low permeability (Caulcott *et al.*, 1984; Yoneyama and Nakae, 1986; Yoshihara *et al.*, 1988) and porins OprC, OprD and protein E1 were shown to form monosaccharide-permeable pores in reconstituted liposome membranes, while OprF, OprG and protein H did not exhibit any pore-forming activity (Yoshihara and Nakae, 1989; Yamano *et al.*, 1990). However, this has been disputed by Nikaido *et al.*, (1991) who suggest that OprF was inadvertently inactivated during purification. Although still considered to be a porin candidate, OprF appeared to be unaltered in this study, in agreement with other workers (Hirai *et al.*, 1987; Yamano *et al.*, 1990).

In this study a dose-dependent loss of OprG was associated with increased resistance. At present the function of OprG in the OM of *P. aeruginosa* is unknown. Chamberland *et al.*, (1989) proposed a correlation between reduction of OprG in the OM and altered cell permeability leading to increased resistance to norfloxacin in *P. aeruginosa*. Fukuda *et al.*, (1990) also noticed a similar reduction in OprG in their norfloxacin-resistant mutant of *P. aeruginosa* PAO. In addition, ciprofloxacin-resistant *P. aeruginosa* derivatives isolated during ciprofloxacin therapy of patients with cystic fibrosis (CF) were shown to have a 90% reduction of OprG in their OM (Chamberland *et al.*, 1989). In this study the CF

clinical isolate was not associated with a decrease in OprG expression. However, although the MICs for the variants were found to be constant after one passage in antibiotic-free medium, the OMP profiles previously observed in the resistant isolates reverted to those shown by the initial susceptible isolate PAO1. Since the CF isolate was obtained on antibiotic-free medium this may explain why OprG expression was unaltered.

This study implicates a reduction of OprG as a possible mechanism of OM permeability modulation in the expression of fluoroquinolone resistance. However, since OprG is not a major OMP and the change observed by other workers (Chamberland *et al.*, 1989; Fukuda *et al.*, 1990) is not a complete loss of the protein, such modification may have been overlooked by other investigators. In fact in a previous report which related permeability resistance to norfloxacin to the acquisition of a new 54 kDa OMP (Hirai *et al.*, 1987), it also appears that OprG may have been reduced in quantity. Similarly, loss of OprG can be observed in the aminoglycoside resistant strains of *P. aeruginosa* with OprG expression restored in the reverted susceptible strain (Gilleland *et al.*, 1989). Shand *et al.*, (1988a) demonstrated that when cells of *P. aeruginosa* strain PAO1 normally sensitive to 10 units.ml⁻¹ of polymyxin, were trained to resist polymyxin at 6000 units.ml⁻¹, OprG expression was diminished.

Overproduction of the divalent cation-regulated OprH of *P. aeruginosa* is associated with resistance to polymyxin, gentamicin and other aminoglycosides, and EDTA (Nicas and Hancock, 1980, 1983a). OprH is believed to replace divalent cations at polyphosphate binding sites on LPS, which stabilise negatively-charged groups in the lipid A and the core regions of LPS. The basic nature of the deduced amino acid sequence is consistent with this role (Bell and Hancock, 1989). Replacement of divalent cations in this way is thought to block or reduce the self-promoted uptake route by polycationic antibiotics (Nicas and Hancock, 1980), although it has been shown that this mechanism is not the sole source of resistance (Shand *et al.*, 1988a).

A reduction in OprD was exhibited by the ciprofloxacin-resistant variants. OprD-deficiencies have been associated with expression of resistance to β -lactams, quinolones and chloramphenicol in *P. aeruginosa* mutants isolated after chemical mutagenesis (Yamano *et*

al., 1990). Latamoxef-resistant mutants showed decreased production of OprC, OprD, proteins E1 and E2 and cross-resistance to quinolones. At least one of these proteins is thought to play an important role in OM permeability, perhaps by functioning as a porin (Yamano *et al.*, 1990). Three of these four proteins C, D2 and E1 are apparently identical to proteins C, D and E, which have been shown to exhibit pore-forming activities (Yoshihara and Nakae, 1989). *P. aeruginosa* mutants resistant to the broad spectrum carbapenem β -lactam imipenem have been shown to lack an OMP of molecular weight 45 kDa to 46 kDa (Quinn *et al.*, 1986; Buscher *et al.*, 1987; Trias *et al.*, 1989; Gotoh and Nishino, 1990; Trias and Nikaido, 1990b). This protein has been identified as OprD which has the characteristics of a substrate-selective porin i.e. one with a specific binding site for imipenem over other β -lactams based on the lack of cross-resistance of OprD mutants to other β -lactams (Quinn *et al.*, 1986). Michea-Hamzhepour *et al.*, (1991) suggest that porin D2 may catalyse facilitated diffusion of the quinolone sparfloxacin, as it does for imipenem. The results also indicate that for crossing the OM sparfloxacin can use alternative pathways. Several proteins are candidates for this pathway, notably porins E2 (Yamano *et al.*, 1990) and OprG (Chamberland *et al.*, 1989).

It is therefore possible that decreased amounts of more than one protein might be necessary for antibacterial resistance. If more than one protein could function as a porin for antibacterials, lack of only one protein would not necessarily cause a high level of resistance, i.e producing low level resistance (Yamano *et al.*, 1990).

The increase in OprH noted in this study may be attributable in part to the chelating activity of ciprofloxacin, although stoichiometric calculations indicate that Mg^{2+} ions were available in the free form. The ciprofloxacin sensitivity data studying the effect of Mg^{2+} ions in the assay medium suggest that ciprofloxacin availability may be compromised by high divalent cation concentration. Excess Mg^{2+} prevents quinolones from chelating LPS-associated Mg^{2+} and creating hydrophobic patches on the cell surface and therefore limits their diffusion across the OM to the porin pathway. Smith (1989b) has observed a similar effect and has suggested that co-administration of ciprofloxacin with Al^{3+} salts and other antacids may cause treatment failure in patients. Recently, it was proposed that quinolones interact

with the OM of *E. coli* as chelating agents (Chapman and Georgopapadakou, 1988). They would create displacement of membrane-bound Mg^{2+} exposing hydrophobic domains on the OM, through which they would penetrate the bacterial cells. Therefore, quinolones may be able to penetrate the OM of all Gram-negative bacteria by this additional non-porin pathway (Chapman and Georgopapadakou, 1988; Bryan and Bedard, 1991).

LPS is a major constituent of the OM of Gram-negative bacteria and has essential roles in the structure and function of the membrane (Kropinski *et al.*, 1985). In addition, LPS is an important virulence factor (Cryz *et al.*, 1984) and therefore has a role in pathogenesis. Hirai *et al.*, (1986a) noted that the MIC of quinolones was decreased in lipopolysaccharide-deficient (rough) mutants as compared with the wild type and the size of the decrease correlated with the hydrophobicity of the quinolone. These observations suggested that the passage of quinolones through the OM was not limited to porins. Chamberland *et al.*, (1989) and Legakis *et al.*, (1989) have measured variations in LPS structure for quinolone resistant variants, including truncation and addition of intermediate length bands. The outer leaflet of the OM phospholipid bilayer contains anionic LPS molecules stabilised by divalent cations such as Mg^{2+} (Nicas and Hancock, 1983a). LPS changes observed in the *in-vivo*-selected resistant mutants may reflect a change in divalent cation saturation of the OM that would in turn affect the non-porin pathway of quinolone uptake. No significant changes in LPS structure were found in this study by silver-stained SDS-PAGE, which is in agreement with Chamberland *et al.*, (1989) for their *in-vitro* resistant isolates and with Hirai *et al.*, (1987). In addition, cells grown under high and low iron conditions demonstrated similar LPS profiles. However, in this study the KDO:protein ratio in the OM decreased 5-fold in the ciprofloxacin-resistant cells, indicating perturbed expression of LPS. This finding suggests that the involvement of LPS in the expression of ciprofloxacin resistance is likely.

KDO content, a marker compound for LPS has been found to vary with growth rate of the cultures, slower growing cells containing more than the faster growing ones (Gilbert and Brown, 1978). In contrast to the results found here, it has been suggested that an increased KDO content might result in decreased uptake of 3- and 4-chlorophenol (Gilbert and

Brown, 1978). However, this observation does not preclude the possibility that acquisition of resistance may also result from more subtle changes in LPS composition, or from variations in envelope proteins associated with the LPS (Ames *et al.*, 1974).

Estimates of surface hydrophobicity of the ciprofloxacin resistant variants showed a decrease under both high and low iron conditions. In contrast, Chapman and Georgopapadakou (1988) and Chamberland *et al.*, (1990) showed an increase in cell surface hydrophobicity with *E. coli* and *P. aeruginosa*, respectively. The reason for this is unclear, but might relate to differences in the methods of determination (Loh *et al.*, 1984; Hirai *et al.*, 1986b; Fett, 1985). Dillon *et al.*, (1986) recommend a range of assays to be employed for reliable assessment of hydrophobicity, since reliance on a single method may produce conflicting results, as observed here. Hydrophobicity reflects alterations of the cell surface, possibly with respect to surface appendages or regulation of protein biosynthesis (Allison *et al.*, 1990). Chamberland *et al.*, (1990) noticed that OprF was completely absent in their *P. aeruginosa* resistant mutant, proposing that OprF may have been replaced by lipid material accounting for the increase in hydrophobicity. In this study OprF was found to be unaltered. Hydrophobicity is not only dictated by growth rate of the organism and the nature of the envelope, but also by the species (Domingue *et al.*, 1989; Allison *et al.*, 1990) maybe accounting for the difference observed between *E. coli* and *P. aeruginosa*.

Despite reduced uptake of norfloxacin being demonstrated by Chamberland *et al.*, (1989) in three clinically-isolated resistant strains, altered cell permeability was thought to contribute modestly to the expression of quinolone resistance in the isolates. Mutations rendering DNA synthesis (DNA gyrase) more resistant to quinolones would be the primary determinant of resistance in these *in vivo*-isolated mutants (Chamberland *et al.*, 1989; Masecar *et al.*, 1990). For *in-vitro*-selected mutants Chamberland *et al.*, (1989) suggested that quinolone resistance was due to an alteration in OM permeability reducing drug entry, probably owing to changes in porin channels. This type of resistance being associated with resistance to other antimicrobial agents, as found in this study.

Plasmid-mediated resistance is unlikely to be responsible for the observed changes (Munshi *et al.*, 1987). Two other mechanisms might account for the emergence of resistance. One

is diminished affinity of the drug for the target enzyme topoisomerase II (DNA gyrase; Hooper *et al.*, 1986, 1987). The resistant variants were not tested for inhibition of DNA gyrase supercoiling activity by quinolones. Resistance to quinolones can involve an alteration in the subunit A protein of DNA gyrase (Hooper *et al.*, 1987; Inoue *et al.*, 1987; Munshi *et al.*, 1987; Neu, 1988). Such an alteration might have occurred on passage through antibiotic-free medium when alterations in OMP profiles were lost, but resistance maintained for five passages. This supports the notion that a modification of the intracellular target site of the quinolones, the DNA gyrase, was the primary determinant of resistance in these *in vitro* selected resistant isolates. With altered OMPs a lower affinity of binding of the drug to surface receptors and/or a lower permeability of the drug must be considered as mediators of resistance. Since OMP changes reflect gene function, the primary cause could be either mutational or a nonlethal drug-induced chromosomal defect. Inhibition of transcription of genes owing to impaired supercoiling of bacterial DNA is a possibility, suggested by simultaneous changes of several OMPs some not associated with drug resistance (Daikos *et al.*, 1988). It is therefore, not possible to assign a specific role of the OMP for impaired cellular permeability to ciprofloxacin as has been shown for *E. coli* norfloxacin-resistant mutants (Hirai *et al.*, 1986b; Hooper *et al.*, 1986; Aoyama *et al.*, 1987). Consequently it is reasonable to suggest that defective OM permeability operates synergistically with probably an altered DNA gyrase, affording a high level of ciprofloxacin resistance (Legakis *et al.*, 1989).

In summary, these data indicate that expression of OprG can be modulated by growth rate/phase, availability of iron and by the presence of ciprofloxacin in the growth medium. Since a reduced copy number of OprG correlated with increased resistance to different antibiotic groups and decreased entry of drug (Chamberland *et al.*, 1989), the results of these experiments would support the hypothesis that OprG has either porin channel-forming activity or that reduction of this protein together with alteration in the expression of other porins, could alter their function. Whatever its true function(s) the very presence of OprG in the OM may be enough to affect the cell's response to its environment.

RESULTS CHAPTER 5

5. EFFECT OF THE CHEMOTHERAPEUTIC AGENT MITOMYCIN C ON SURFACE PROPERTIES OF *P. aeruginosa*.

5.1 INTRODUCTION.

Research in the laboratory at Aston university has contributed to the finding that IRMPs, integral components of the iron-uptake systems of Gram-negative bacteria, are powerful immunogens expressed *in vivo* and recognised by the human host's immune system. Bacteria recovered directly from infections of the urinary tract (Lam *et al.*, 1984; Shand *et al.*, 1985), burn wounds (Ward *et al.*, 1988) and the cystic fibrotic lung (Brown *et al.*, 1984; Anwar *et al.*, 1984; Cochrane *et al.*, 1987, 1988) have all been shown to express IRMPs.

It has been suggested that OprG comprises part of a low-affinity iron-uptake system or functions in the uptake of other nutrients (see chapter 4). Sera from cystic fibrosis patients with *Pseudomonas* lung infection have been used in immunoblotting studies where OprG was found to be highly immunogenic (Anwar *et al.*, 1984; Dr.J.West, personal communication). In contrast, strong recognition of OprG was not observed with human burn wound infection (Ward *et al.*, 1988). In cystic fibrosis, *P. aeruginosa* causes a chronic infection which may persist for upwards of 20 years. The bacteria grow in glycocalyx-enclosed micro-colonies embedded in exopolysaccharide and mobile swarmer cells are frequently released resulting in exacerbation of pneumoniae (Costerton *et al.*, 1983). Over the years, *P. aeruginosa* exposes many of its antigens to the immune system, thus stimulating the production of high titers of antibodies to numerous bacterial components (Hoiby and Axelsen, 1973). This is in contrast to *P. aeruginosa* in burn wounds, which is characterised by frequently rapid dissemination causing life-threatening septicaemia. The observed immune response is thus against the acute infection episode.

To extend the evidence on the recognition of these important antigenic determinants by the host's immune system, another infection situation was investigated, namely the

immunocompromised leukaemic patient. There appears to be a relationship between the type of cancer and the subsequent bacterial infection. *P. aeruginosa* has been isolated in over 50% of leukaemic patients who developed bacterial infection (Bodey, 1986).

The research was divided into two aspects, firstly the effect of the antineoplastic agent (mitomycin C) on the surface properties of *P. aeruginosa* and secondly the immune response and iron status of leukaemic patients throughout an infection episode (chapter 6). There is little information on the antimicrobial activity of antineoplastic agents and no systematic study of this topic appears to have been undertaken. Cytotoxic agents have been known for a long time to have immunosuppressive characteristics, but some of them have been also shown to exhibit antimicrobial activity (Moody *et al.*, 1978). A microorganism not inhibited by these compounds would therefore have a definite advantage in establishing an infection in the cancer patient. One study has compared twenty-one antineoplastic agents against twenty-eight microbial strains. The results show mitomycin C to be the most active compound (Hamilton-Miller, 1984; see section 1.1.1). Therefore, studies focused on this compound. Surface properties of *P. aeruginosa* were assessed by SDS-PAGE of bacterial OMPs, immunoblotting, bacterial adherence to hydrocarbon (BATH) and analysis of LPS.

5.2 DETERMINATION OF SUB-INHIBITORY CONCENTRATIONS OF MITOMYCIN C TO *P. aeruginosa*.

The growth kinetics of *P. aeruginosa*, grown under high and low iron conditions in the presence and absence of sub-inhibitory concentrations (sub-MICs) of mitomycin C was investigated (see section 3.4.1). Shown are strains PAO1 (Fig 5.2.1 and 5.2.2; MIC= 1 μ g.ml⁻¹), 6750 (Fig 5.2.3 and 5.2.4; MIC= 1 μ g.ml⁻¹), M7 (Fig 5.2.5 and 5.2.6; MIC= 1 μ g.ml⁻¹) and AK1012 (Fig 5.2.7 and 5.2.8; MIC= 1 μ g.ml⁻¹), grown under CDM+Fe and CDM-Fe, respectively. These strains were selected because PAO1 is a smooth strain of *P. aeruginosa*, while AK1012 is a rough mutant derived from *P. aeruginosa* PAO1. Strains 6750 and its mucoid variant M7 are both semi-rough. Under the conditions described, the doubling time and growth rate of strains, PAO1= 60min, 1h⁻¹; 6750= 72min, 1.2h⁻¹; M7= 78min, 1.3h⁻¹; and AK1012= 90min, 1.5h⁻¹. The growth rate of each of these strains was not affected by the media iron concentration. Early stationary phase cells were taken at

Fig 5.2.1

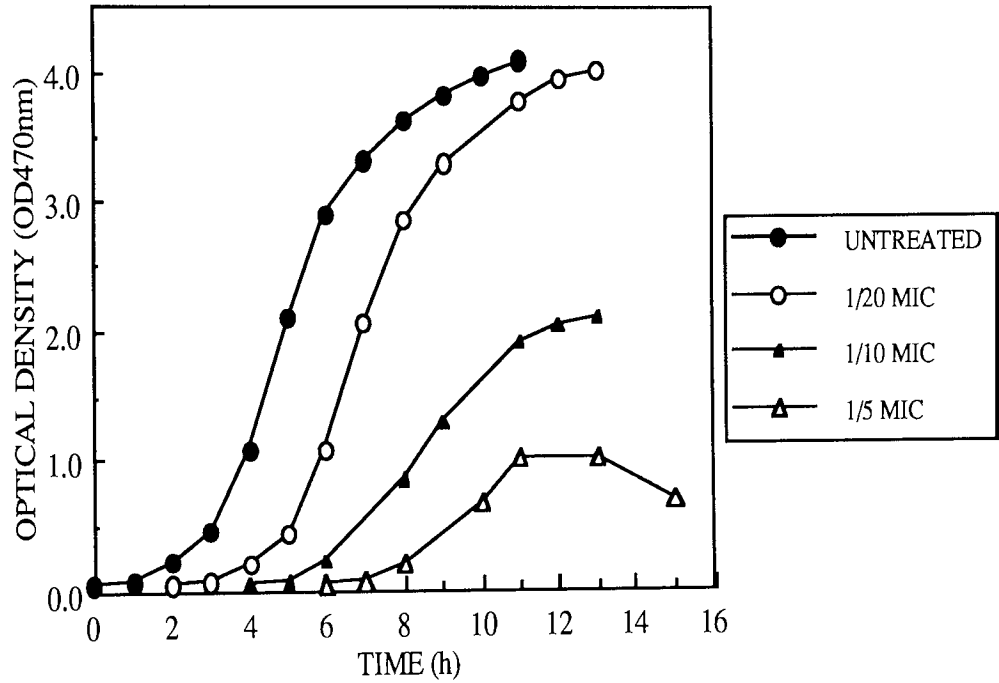
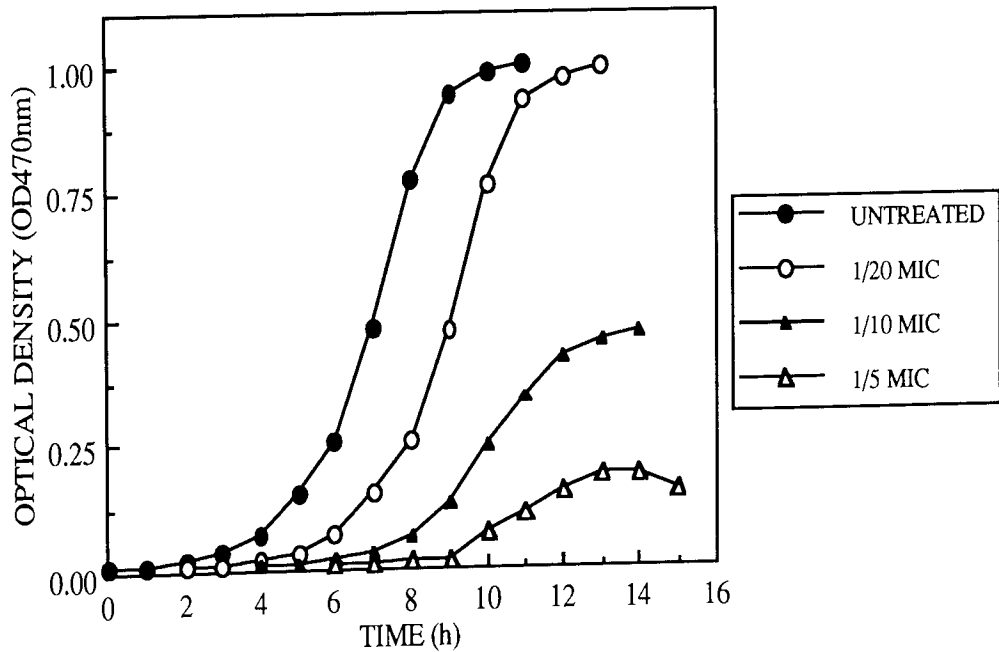


Fig 5.2.2



The effect of mitomycin C on the growth kinetics of *P. aeruginosa* PAO1 under iron-plentiful (Fig 5.2.1) and iron-depleted (Fig 5.2.2) CDM batch culture. The curves have been offset for clarity.

Fig 5.2.3

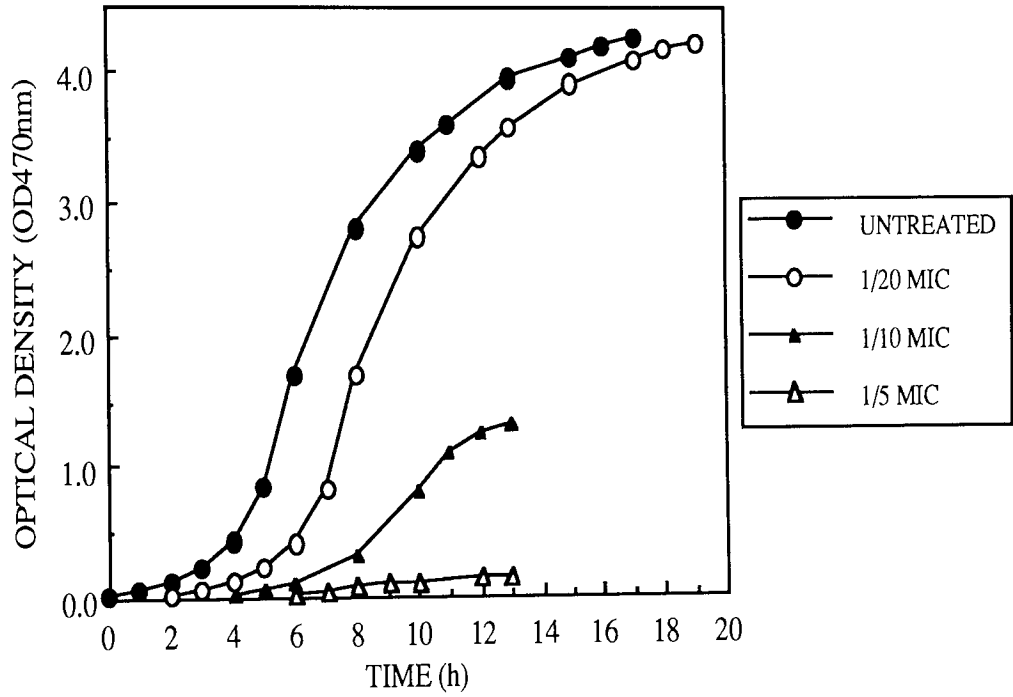
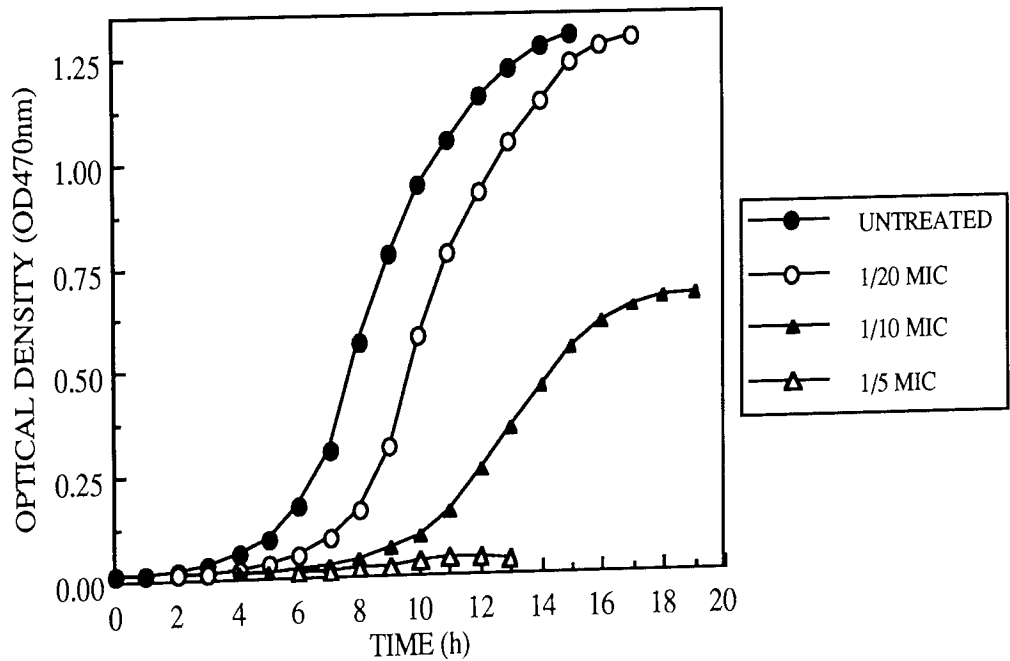


Fig 5.2.4



The effect of mitomycin C on the growth kinetics of *P. aeruginosa* 6750 under iron-plentiful (Fig 5.2.3) and iron-depleted (Fig 5.2.4) CDM batch culture. The curves have been offset for clarity.

Fig 5.2.5

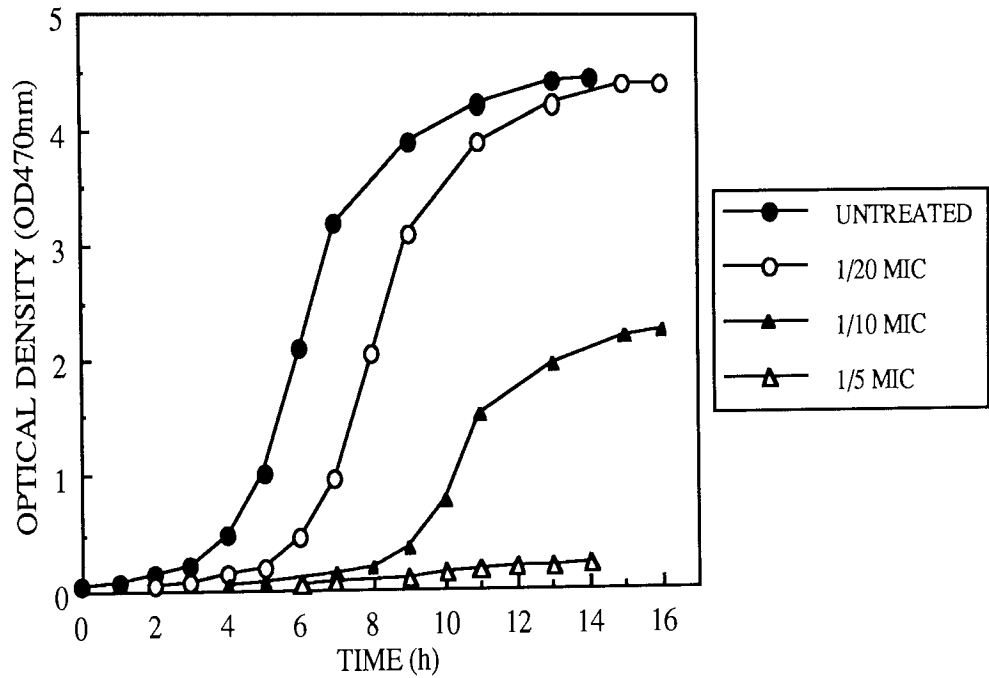
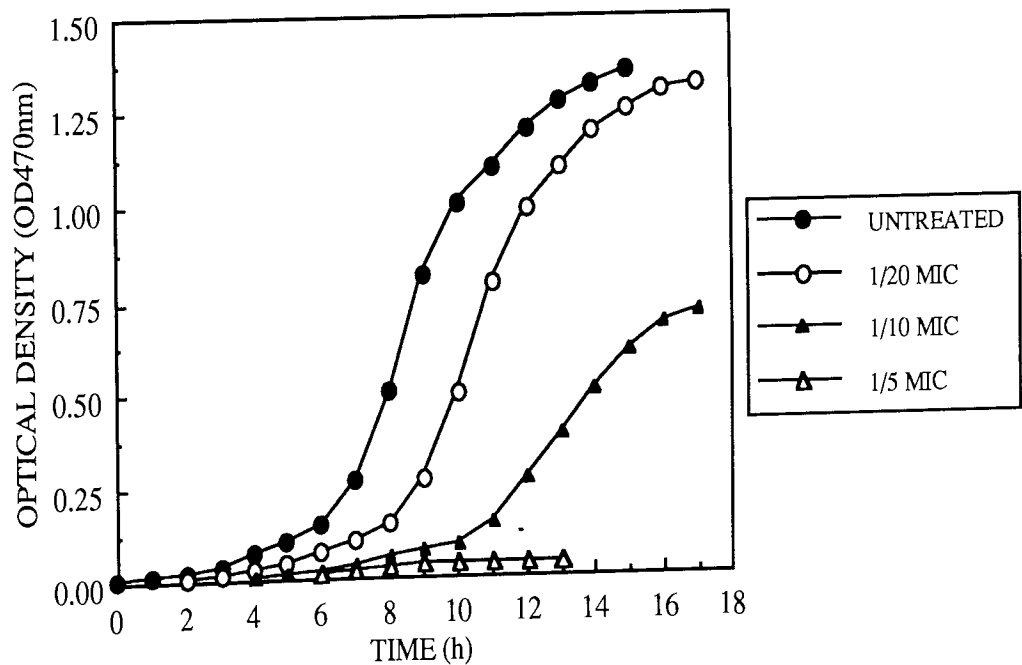


Fig 5.2.6



The effect of mitomycin C on the growth kinetics of *P. aeruginosa* M7 under iron-plentiful (Fig 5.2.5) and iron-depleted (Fig 5.2.6) CDM batch culture. The curves have been offset for clarity.

Fig 5.2.7

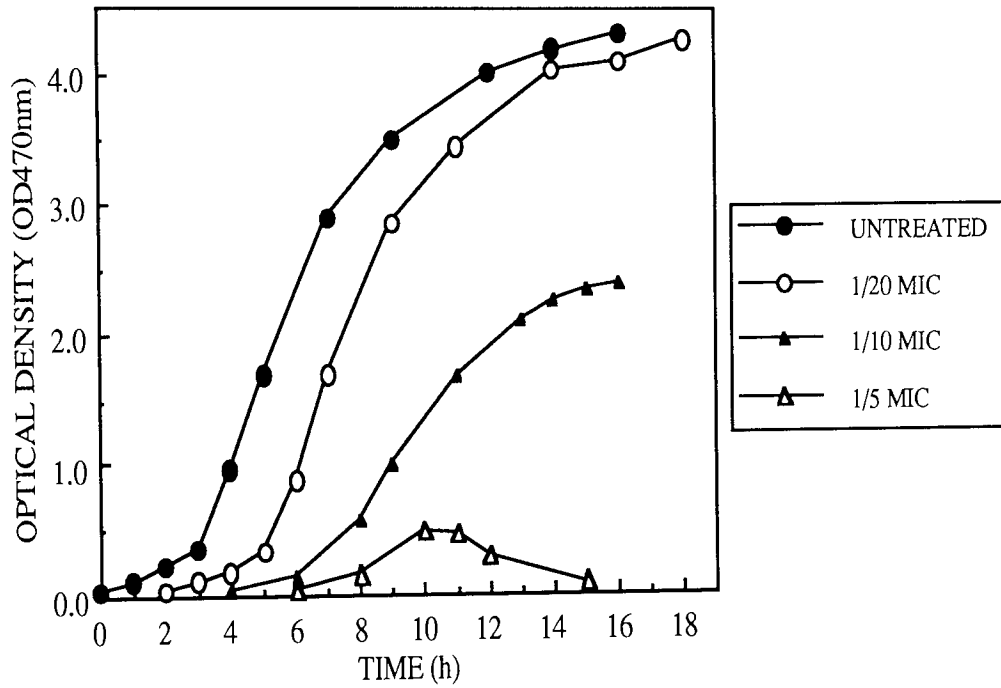
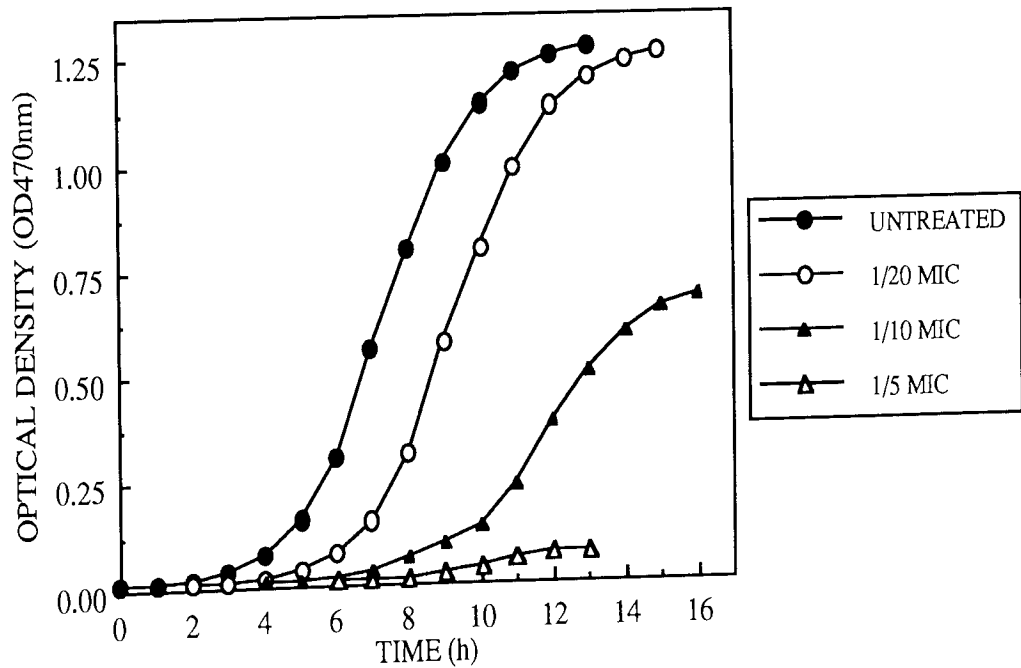


Fig 5.2.8



The effect of mitomycin C on the growth kinetics of *P. aeruginosa* AK1012 under iron-plentiful (Fig 5.2.7) and iron-depleted (Fig 5.2.8) CDM batch culture. The curves have been offset for clarity.

OD₄₇₀ 3.5, 0.85 for PAO1; OD₄₇₀ 3.75, 1.2 for 6750; OD₄₇₀ 3.9, 1.2 for M7; and at OD₄₇₀ 3.75, 1.15 for AK1012, under CDM+Fe and CDM-Fe conditions, respectively. The results show that in the presence of 1/20 MIC (0.05µg.ml⁻¹) of mitomycin C, the lag time, doubling time and final OD remained unchanged for all strains compared to their appropriate untreated controls. The media iron concentration did not appear to affect the sensitivity of the organisms to mitomycin C. At 1/10 and 1/5 MIC an increased lag time and a reduced growth rate and final OD is observed. In addition, the bacteria were clumped and filament formation was noted. Subsequently, 1/20 MIC (0.05µg.ml⁻¹) mitomycin C was used for further investigation.

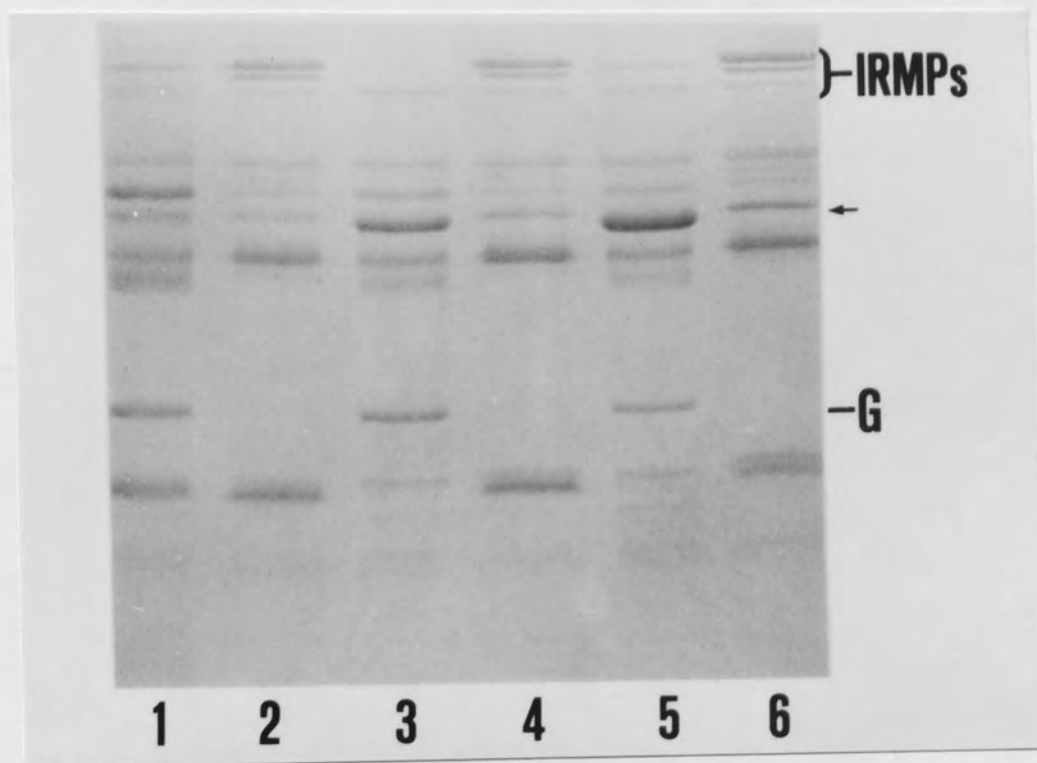
5.3 ANALYSIS OF OMP PROFILES OF *P. aeruginosa* UNDER IRON-PLENTIFUL AND IRON-DEPLETED CONDITIONS, IN THE PRESENCE AND ABSENCE OF MITOMYCIN C.

The effect of sub-MICs of mitomycin C on the OMP profiles of *P. aeruginosa* grown under high and low iron conditions and harvested at early stationary phase was investigated by SDS-PAGE. Shown are strains PAO1 (Fig 5.3.1), 6750 (Fig 5.3.2), M7 (Fig 5.3.3.1 and 5.3.3.2) and AK1012 (Fig 5.3.4). From the resultant profiles OprG was repressed by low iron and induced by high iron concentrations in the media and expression did not appear to be influenced by mitomycin C. Strains 6750 and M7 show the acquisition of a new OMP (molecular weight 32 kDa; marked with arrow), the expression being particularly strong for 6750 and is present regardless of media iron concentration. PAO1 and AK1012 under iron-replete conditions, show loss of protein H2 (OprL) together with an increase in protein E (OprE; marked with arrow).

5.4 ANTIBODY RESPONSE IN SERA FROM A LEUKAEMIC PATIENT INFECTED WITH *P. aeruginosa*.

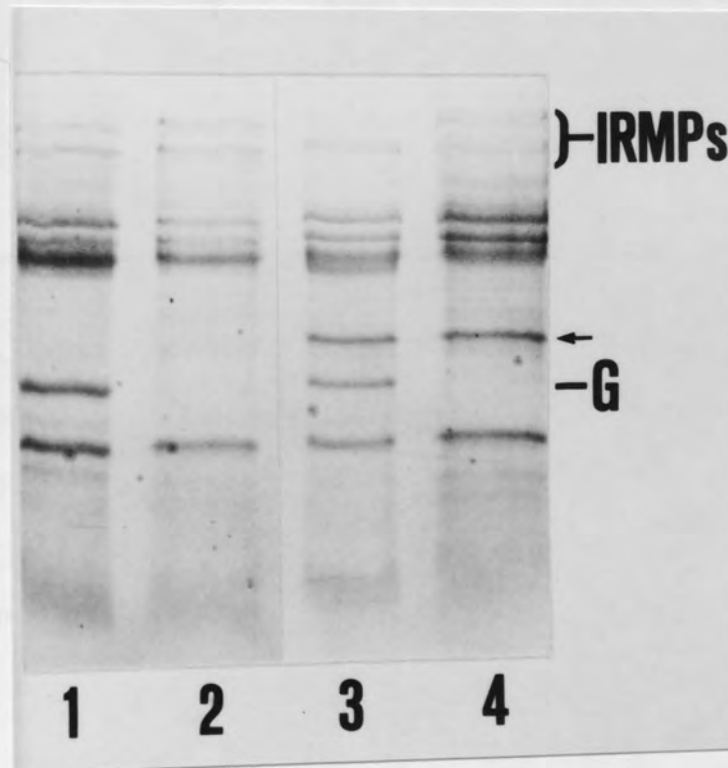
Immunoblotting combines the high resolution of SDS-PAGE with a sensitive solid phase immunoassay, such that the antigenic properties of separated OM components can be investigated (Towbin and Gordon, 1984). Sera from a leukaemic patient infected with *P. aeruginosa* were absorbed with extracted LPS from strain 6750, prior to use in

Fig 5.3.1



Coomassie blue-stained SDS-PAGE, showing the effect of iron and sub-MICs of mitomycin C on the OMP profiles of *P.aeruginosa* PAO1. Lanes 1, 3 and 5 show CDM+Fe, while lanes 2, 4 and 6 are CDM-Fe conditions. Lanes 3 and 4 show PAO1 grown in $0.01\mu\text{g.ml}^{-1}$ and lanes 5 and 6 show $0.05\mu\text{g.ml}^{-1}$ mitomycin C. The arrow indicates the increase in OprE expression.

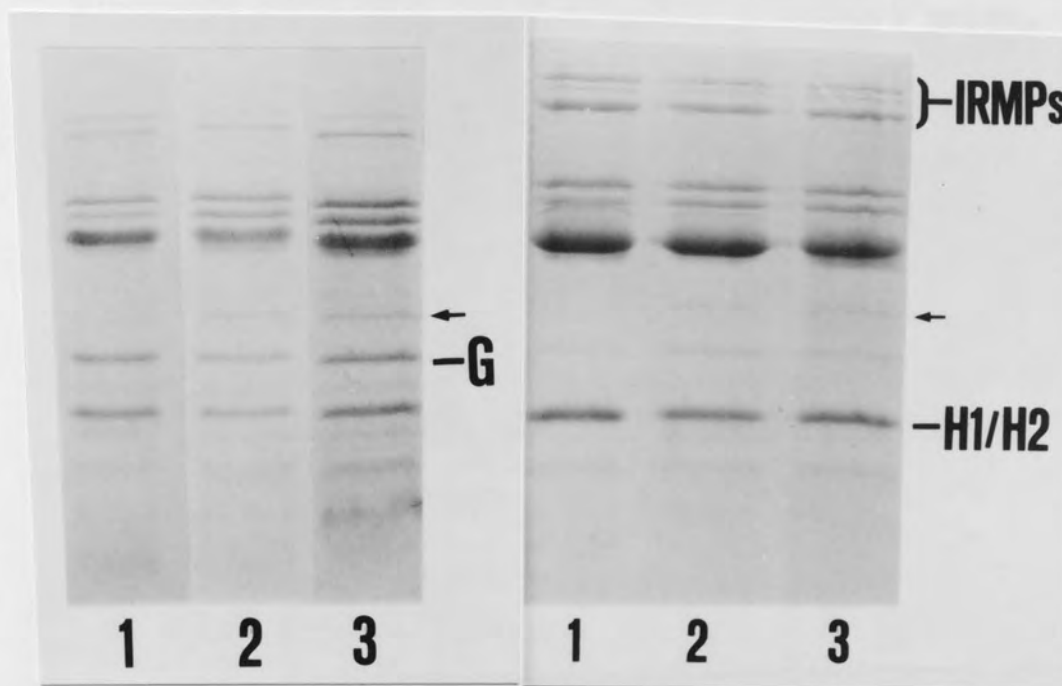
Fig 5.3.2



Coomassie blue-stained SDS-PAGE, showing the effect of iron and sub-MIC of mitomycin C on the OMP profiles of *P. aeruginosa* 6750. Lanes 1 and 3 show CDM+Fe, while lanes 2 and 4 show CDM-Fe conditions. Lanes 3 and 4 show 6750 grown in $0.05\mu\text{g.ml}^{-1}$ mitomycin C. The arrow represents the acquisition of a new OMP.

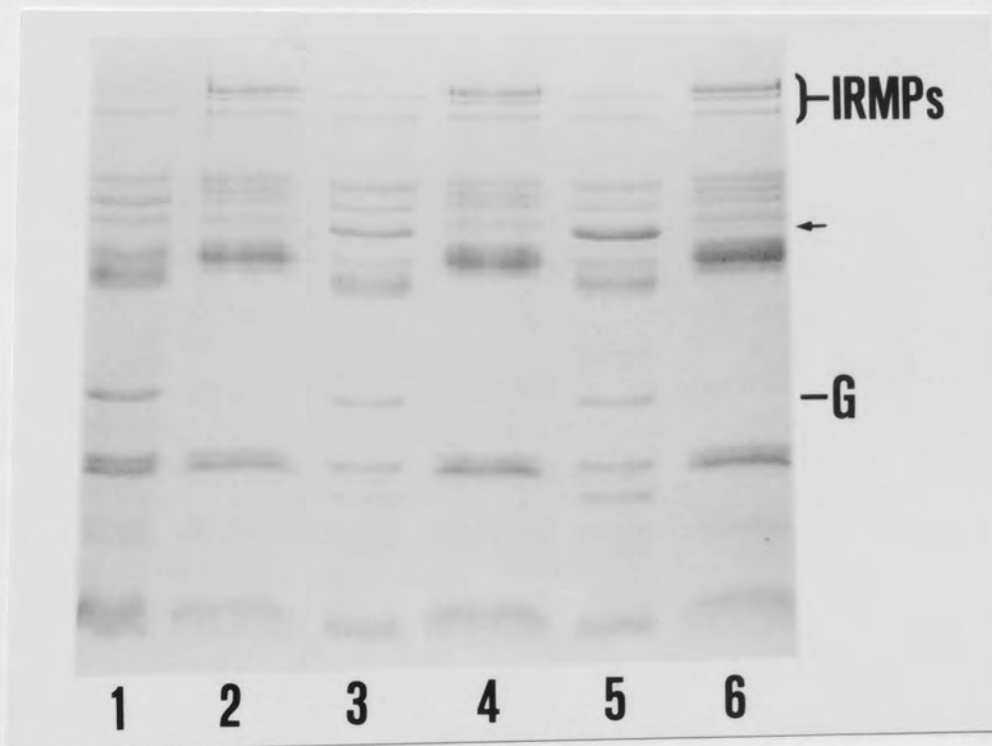
Fig 5.3.3.1

Fig 5.3.3.2



Coomassie blue-stained SDS-PAGE showing the effect of iron and sub-MICs of mitomycin C on the OMP profiles of *P. aeruginosa* M7. Figure 5.3.3.1 shows M7 grown in CDM+Fe with $0.01\mu\text{g}\cdot\text{ml}^{-1}$ (lane 2) and $0.05\mu\text{g}\cdot\text{ml}^{-1}$ mitomycin C (lane 3). Figure 5.3.3.2 shows M7 grown in CDM-Fe with $0.01\mu\text{g}\cdot\text{ml}^{-1}$ (lane 2) and $0.05\mu\text{g}\cdot\text{ml}^{-1}$ mitomycin C (lane 3). The arrows represent the acquisition of a new OMP.

Fig 5.3.4



Coomassie blue-stained SDS-PAGE, showing the effect of iron and sub-MICs of mitomycin C on the OMP profiles of *P. aeruginosa* AK1012. Lanes 1, 3 and 5 show CDM+Fe, while lanes 2, 4 and 6 show CDM-Fe conditions. Lanes 3 and 4 show AK1012 grown in $0.01\mu\text{g.ml}^{-1}$ and lanes 5 and 6 show $0.05\mu\text{g.ml}^{-1}$ mitomycin C. The arrow indicates the increase in OprE expression.

immunoblotting to avoid immunodetection of antigens due to co-migrating LPS (Poxton *et al.*, 1985). The separated OMPs of 6750 (Fig 5.3.2), were electrophoretically transferred to NC paper and an amido black stain carried out to determine qualitative transfer of protein bands. Complete transfer of the SDS-PAGE separated proteins was confirmed by staining the polyacrylamide gel after immunoblotting, with Coomassie blue stain. The amido black stain is shown only for cells cultivated under high iron concentrations, indicated by the presence of OprG (Fig 5.4, lane 5).

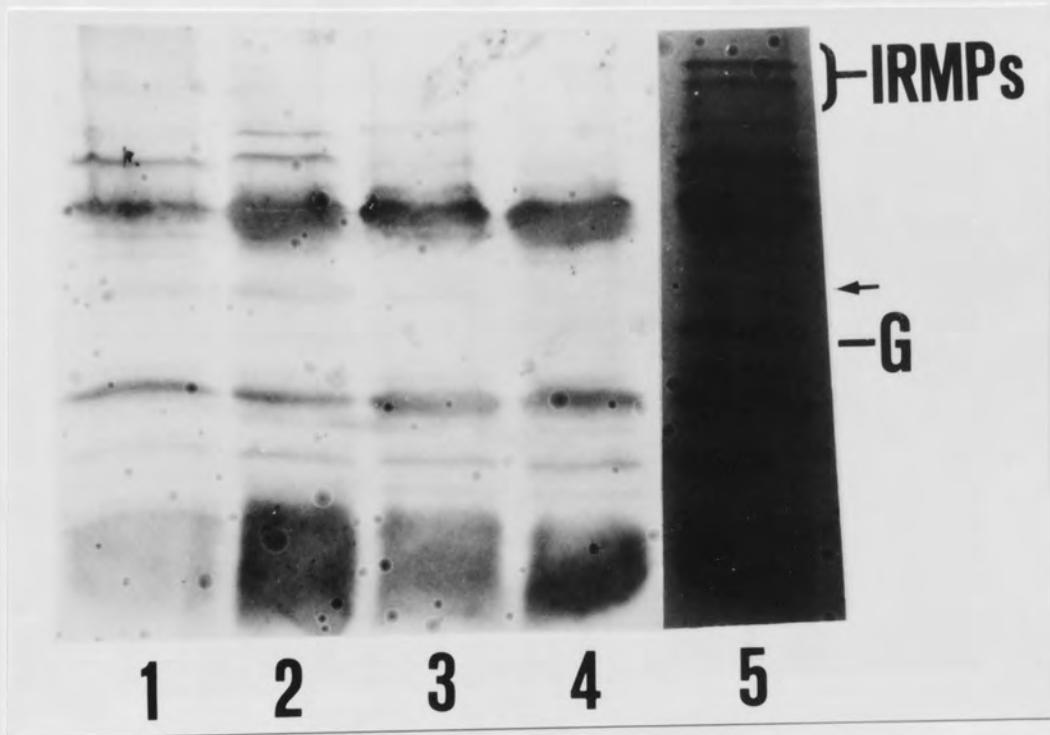
Antigens of *P. aeruginosa* 6750 recognised by IgG antibodies in the serum from a leukaemic patient, 14 days after initial sign of infection are shown (Fig 5.4). Interestingly, there was no recognition of IRMPs (predominantly the 75-85 kDa proteins) in cells grown in *in vitro* conditions of iron-depletion (lanes 1 and 3). Furthermore, no response to OprG was detected in cells grown under iron-plentiful conditions (lanes 2 and 4). Serum IgG antibodies showed a strong reaction to OprF and OprL and additionally to OprE in the mitomycin C-treated cells (lanes 1 and 2). The additional band expressed above OprG (Fig 5.3.2, lanes 3 and 4) was only faintly detected (marked by arrow).

5.5 EFFECT OF MITOMYCIN C ON THE SURFACE HYDROPHOBICITY OF *P. aeruginosa* GROWN IN THE PRESENCE AND ABSENCE OF IRON.

A number of methods for studying hydrophobic interactions of cells have been reported in the literature. No single method adequately describes cell surface hydrophobicity since the experimental conditions employed influence the observed hydrophobic interactions to some degree. Bacterial adherence to hydrocarbon (BATH) was used to evaluate the hydrophobicity. It is a simple and rapid quantitative assay, but the high interfacial tension can cause bacteria to accumulate at the interface between the organic and aqueous phases used in this technique (Kjelleberg and Hermansson, 1984).

The surface hydrophobicity of *P. aeruginosa* PAO1 grown to early stationary phase under iron-plentiful and iron-depleted conditions, in the presence and absence of mitomycin C, was investigated (see section 3.4.10). The mean of ten determinations per sample were carried out on two separate occasions. The results were recorded as the percentage

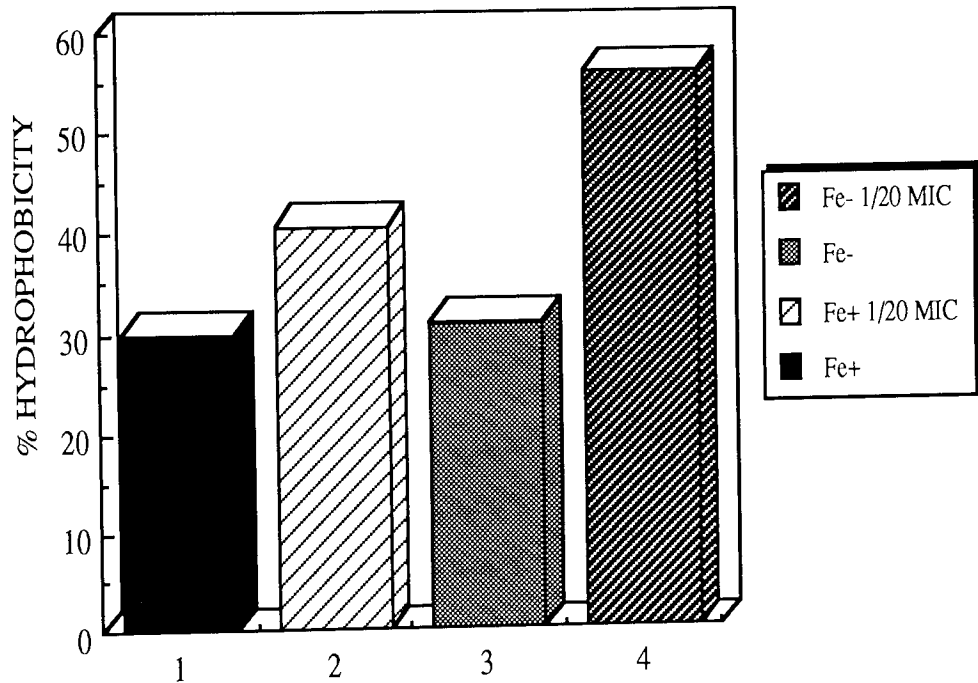
Fig 5.4



Immunoblot of the OMPs described in figure 5.3.2 of 6750 probed with serum from a leukaemic patient infected with *P. aeruginosa*, followed by peroxidase-labelled anti-human IgG. Lanes 2 and 4 show CDM+Fe and lanes 1 and 3 CDM-Fe conditions. Lanes 1 and 2 show 6750 grown in $0.05\mu\text{g.ml}^{-1}$ mitomycin C and lane 5 is the amido black stain.

Fig 5.5

The effect of sub-MIC of mitomycin C on the surface hydrophobicity of *P. aeruginosa* PAO1 in iron-pleteful (lanes 1 and 2) and iron-depleted (lanes 3 and 4) CDM batch culture.



absorbance of the aqueous phase after treatment, relative to the initial absorbance of the bacterial suspension. Figure 5.5 shows that the cell surface became significantly more hydrophobic in the presence of mitomycin C (lanes 2 and 4) compared with the untreated cells (lanes 1 and 3). This effect was more pronounced for cells grown in the absence of iron (lane 4). A similar observation has been made by Kadurugamuwa (1985) who used two methods for assessing hydrophobicity, namely contact angles and adherence to hydrocarbons. The surface of *K. pneumoniae* grown in the presence of sub-MICs of cephalosporins were noted to be more hydrophobic than the untreated cells.

5.6 THE EFFECT OF MITOMYCIN C ON LPS FROM *P. aeruginosa* GROWN IN IRON-PLENTIFUL AND IRON-DEPLETED CONDITIONS.

LPS is an important virulence factor of *P. aeruginosa* and the structure of the O-antigen polysaccharide chain plays an important role in determining the outcome of interactions

with host defence mechanisms (Cryz *et al.*, 1984; Engels *et al.*, 1985). The effect of iron and sub-MICs of mitomycin C on the LPS of *P. aeruginosa* was investigated by SDS-PAGE and immunoblotting and analysis of fatty acids, KDO and carbohydrate content.

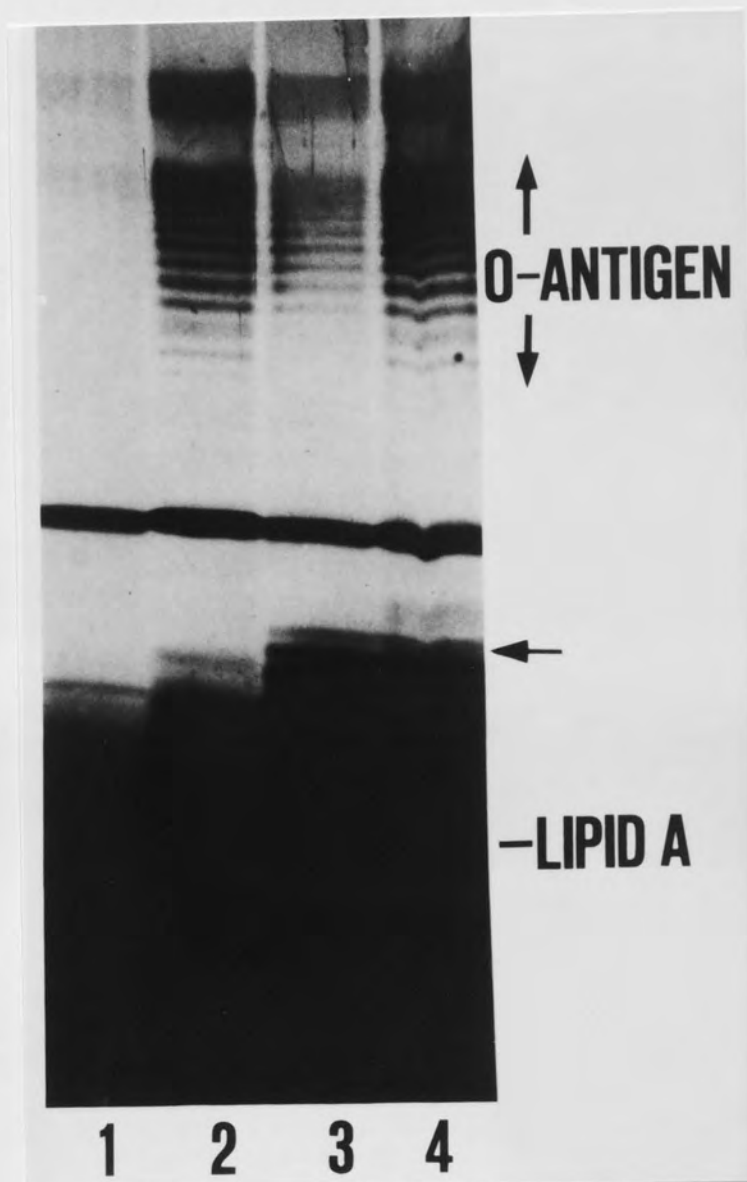
5.6.1 Characterisation of LPS by SDS-PAGE and silver stain.

LPS from five strains of *P. aeruginosa* grown to early stationary phase were examined. The silver-stained LPS profiles of SDS-PAGE-resolved proteinase K digests of whole cells of *P. aeruginosa* PAO1 grown in CDM+Fe (Fig 5.6.1.1) and CDM-Fe (Fig 5.6.1.2) are shown. Two distinct regions are shown, a fast-migrating low molecular weight region, probably representing the core LPS component and a ladder-like series of high molecular weight slow-migrating O-polysaccharide, which represents O-antigen structures with different numbers of repeating units. The LPS includes bands of slightly higher molecular weight. These may represent a proportion of LPS molecules with longer O-polysaccharide side-chains. The LPS for cells cultivated under iron-sufficient (Fig 5.6.1.1) or iron-depleted conditions (Fig 5.6.1.2) demonstrated similar profiles on analysis by SDS-PAGE. This has also been noted by Cochrane (1987) and Ward (1987). The mitomycin C-treated cells (lanes 3 and 4) under high (Fig 5.6.1.1) and low iron conditions (Fig 5.6.1.2) show an induction of low molecular weight bands below the proteinase K band (marked with arrow). This appears to be unaffected by the presence or absence of iron in the media. Subsequently, LPS-PAGE was carried out on cells grown under iron-depleted conditions.

The sample loadings used in this study were chosen to allow complete visualisation of the O-antigen structure. Assuming that LPS represents 10% of the dry weight of whole cells of *P. aeruginosa*, sample loadings were equivalent to approximately 50µg LPS. However, as the results suggest perturbed expression of LPS in the presence of mitomycin C, loadings equivalent to 50µg, 100µg and 150µg dry weight of whole cells were used.

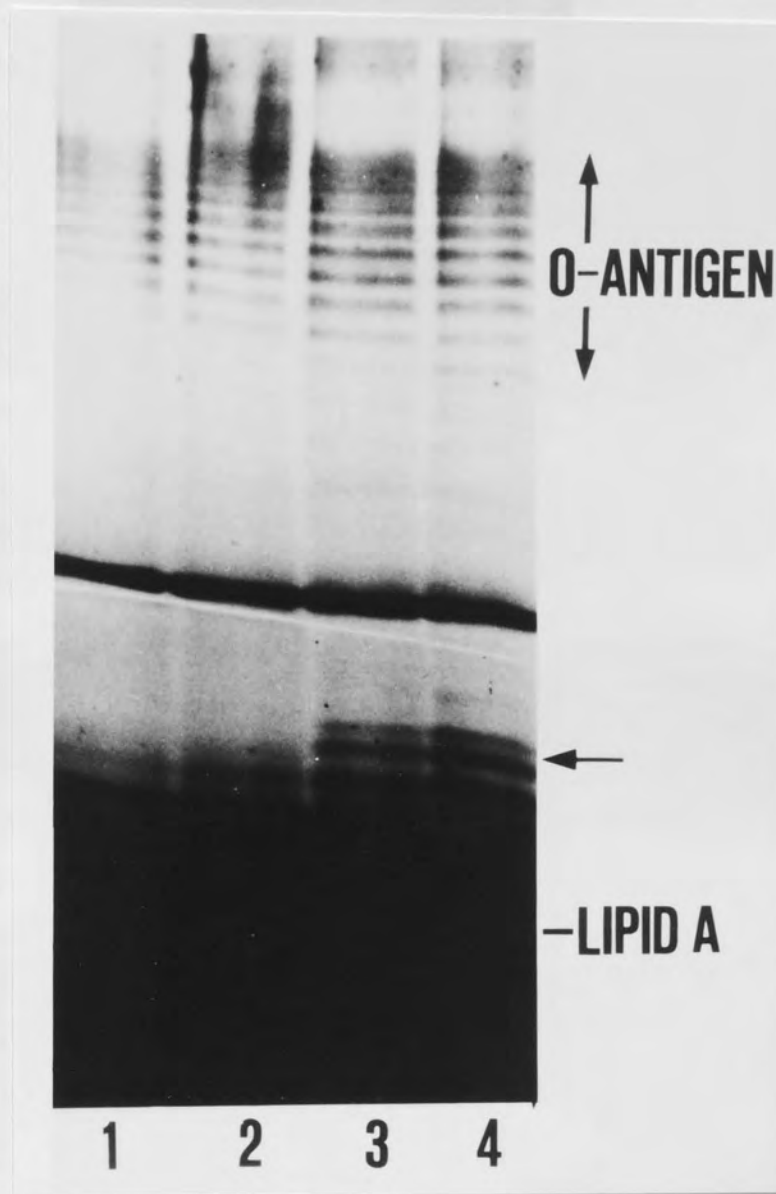
Figure 5.6.1.3 shows the LPS profile of whole cell lysates of *P. aeruginosa* 6750. The cells were grown in CDM-Fe prior to treatment with proteinase K to destroy any proteinaceous material (Hitchcock and Brown, 1983). The results show that in the mitomycin C-treated cells (lanes 2 and 4), a larger proportion of low molecular weight

Fig 5.6.1.1



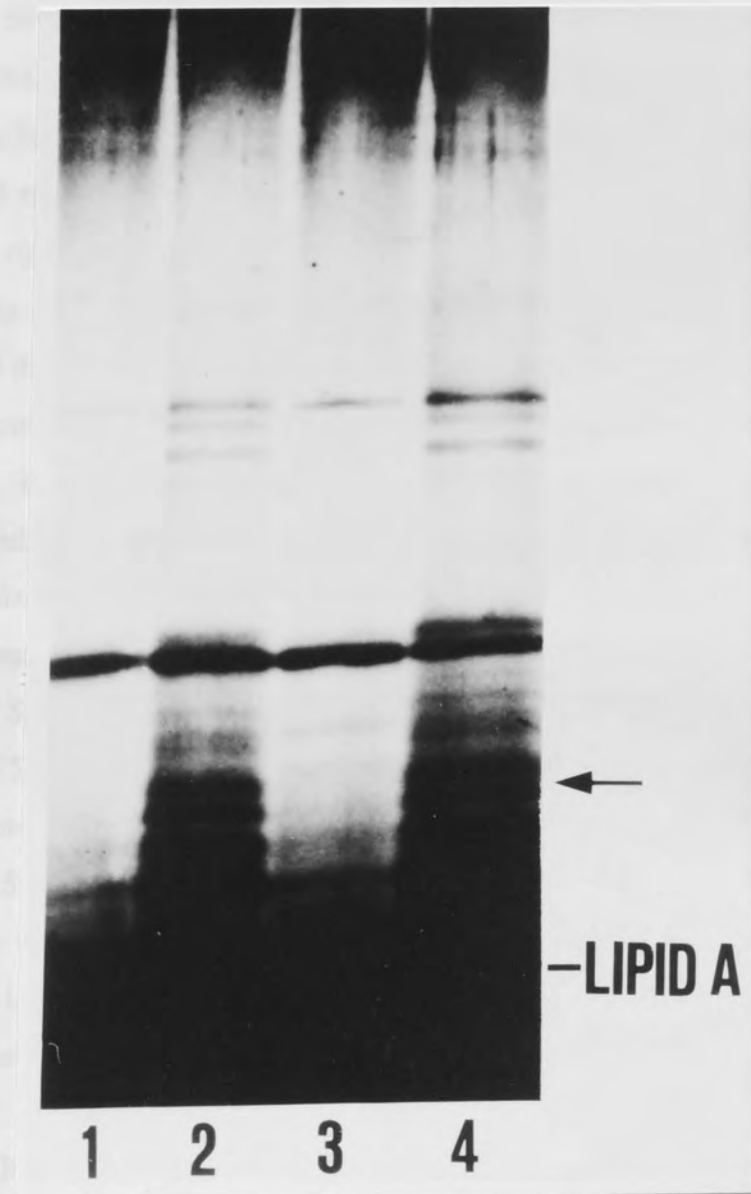
Silver-stained SDS-PAGE profiles of proteinase K digests of whole cell lysates of *P. aeruginosa* PAO1 grown in CDM+Fe (lanes 1 and 2) and with $0.05\mu\text{g}\cdot\text{ml}^{-1}$ mitomycin C (lanes 3 and 4). LPS loadings were equivalent to $50\mu\text{g}$ (lanes 1 and 3) and $100\mu\text{g}$ (lanes 2 and 4) dry weight of cells. Shown are the induction of low molecular weight bands, marked by the arrow.

Fig 5.6.1.2



Silver-stained SDS-PAGE profiles of proteinase K digests of whole cell lysates of *P. aeruginosa* PAO1 grown in CDM-Fe (lanes 1 and 2) and with $0.05\mu\text{g}\cdot\text{ml}^{-1}$ mitomycin C (lanes 3 and 4). LPS loadings were equivalent to $50\mu\text{g}$ (lanes 1 and 3) and $100\mu\text{g}$ (lanes 2 and 4) dry weight of cells. Shown are the induction of low molecular weight bands, marked by the arrow.

Fig 5.6.1.3



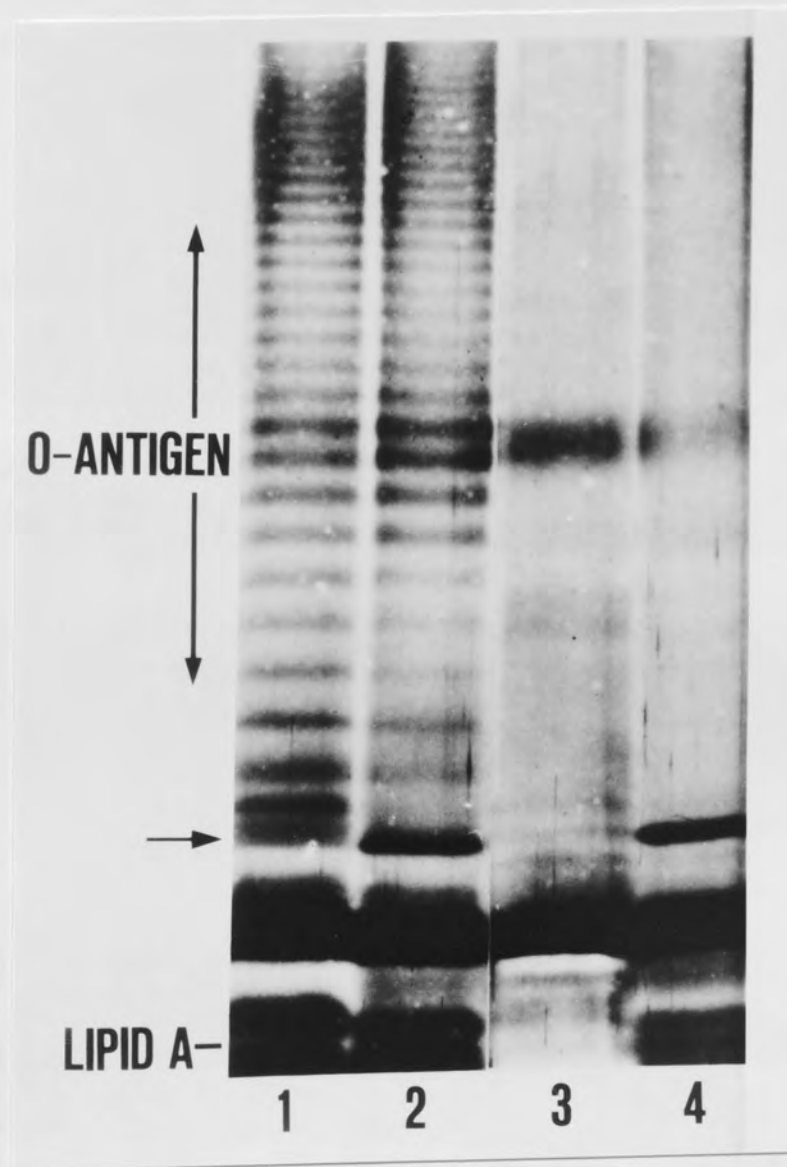
Silver-stained SDS-PAGE profiles of proteinase K digests of whole cell lysates of *P. aeruginosa* 6750 grown in CDM-Fe (lanes 1 and 3) and with $0.05\mu\text{g}\cdot\text{ml}^{-1}$ mitomycin C (lanes 2 and 4). LPS loadings were equivalent to $50\mu\text{g}$ (lanes 1 and 2) and $100\mu\text{g}$ (lanes 3 and 4) dry weight of cells. Shown are the induction of low molecular weight bands, marked by the arrow.

components can be observed (marked with an arrow) similar to that noted with strain PAO1. Greater diversity was found in the pattern of O-antigen material expressed by strain 6750 grown in the presence of mitomycin C.

The SDS-PAGE silver-stained profile of *P. aeruginosa* strains M7 and PAJ grown under iron-depleted conditions in the presence and absence of mitomycin C are shown (Fig 5.6.1.4). The loadings were equivalent to 100µg dry weight of whole cells. PAJ (lanes 1 and 2) contained repeating O-antigen subunits as evident by the ladder-like pattern of bands. The *in vitro* profiles of PAJ show a clearer difference with variations in the interband spacing as well as higher molecular weight material. Kuzio and Kropinski (1983) proposed that a shift to a higher molecular weight could be indicative of a real increase in molecular weight of the side chain or a decrease in the net negative charge on LPS molecules. In addition, Pluschke *et al.*, (1986) noted that altered LPS migration patterns correlated with variations in chemical composition, but further suggested that a combination of size and hydrophobicity may affect the resolution of LPS by SDS-PAGE. Variations in the number of O-polysaccharide repeating units are responsible for the marked heterogeneity of SDS-PAGE profiles of LPS from smooth strains of *Enterobacteriaceae* (Jann *et al.*, 1975; Palva and Makela, 1980; Goldman and Leive, 1980; Hitchcock and Brown, 1983) and of *P. aeruginosa* strains (Wilkinson, 1983; Kropinski *et al.*, 1985). Strain M7 (Fig 5.6.1.4; lanes 3 and 4) appeared to be devoid of O-antigen material. However, when the loadings were increased a faint ladder-like pattern was indeed visualised which is not apparent in this photograph. Again in the presence of mitomycin C (lanes 2 and 4) there is an induction of an additional band (marked with an arrow).

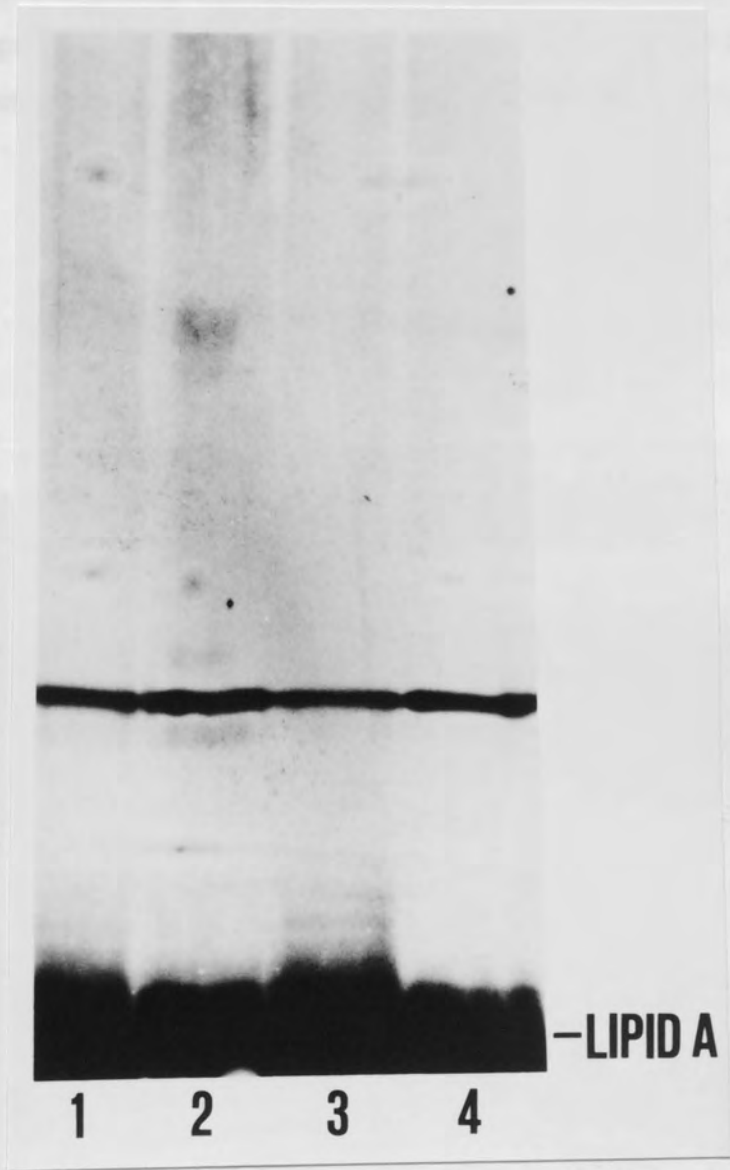
Finally, strain AK1012 was grown under iron-depleted conditions in the presence and absence of sub-MIC of mitomycin C. Shown (Fig 5.6.1.5) are the LPS profiles. When the loadings were increased (150µg) no ladder-like pattern could be visualised. No observable differences could be found for the mitomycin C treated (lanes 3 and 4) and untreated cells (lanes 1 and 2), which is in sharp contrast to the effects previously noted.

Fig 5.6.1.4



Silver-stained SDS-PAGE profiles of proteinase K digests of whole cell lysates of *P. aeruginosa* PAJ (lanes 1 and 2) and M7 (lanes 3 and 4) grown in CDM-Fe (lanes 1 and 3) and with 0.05 μg.ml⁻¹ mitomycin C (lanes 2 and 4). LPS loadings were equivalent to 100 μg dry weight of cells. Shown are the induction of low molecular weight bands, marked by the arrow.

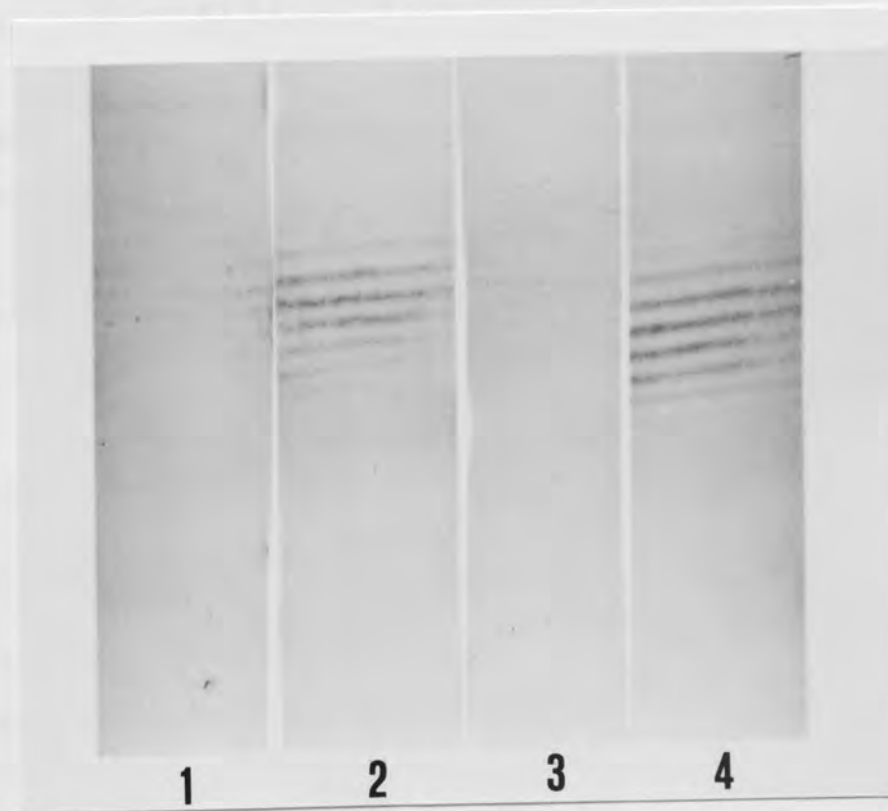
Fig 5.6.1.5



Silver-stained SDS-PAGE profiles of proteinase K digests of whole cell lysates of *P. aeruginosa* AK1012 grown in CDM-Fe (lanes 1 and 2) and with $0.05\mu\text{g}\cdot\text{ml}^{-1}$ mitomycin C (lanes 3 and 4). LPS loadings were equivalent to $50\mu\text{g}$ (lanes 2 and 4) and $100\mu\text{g}$ (lanes 1 and 3) dry weight of cells.

Figure 5.6.1.6 shows the LPS profile of *in-vitro* grown CDM-Fe PAO1 that was revealed by silver staining (Fig. 5.6.1.2) and immunoblotting with rabbit serum hyperimmune to OMs-Fe of *P. aeruginosa* PAO1. The profiles revealed were similar, indicating that the IgG antibodies in the serum reacted with the O-antigen components, but not with the LPS core for both the mitomycin C treated (lane 3 and 4) and untreated cells (lane 1 and 2). Serum from a leukaemic patient infected with *P. aeruginosa* was also used to probe the LPS immunoblot. No detectable reaction was observed (data not shown).

Fig 5.6.1.6



LPS immunoblot of CDM-Fe PAO1 probed with rabbit hyperimmune serum to OMs Fe- of PAO1 followed by peroxidase-labelled anti-human IgG. Lanes 1 and 2 show CDM-Fe PAO1 and lanes 3 and 4 show PAO1 grown in $0.05\mu\text{g.ml}^{-1}$ mitomycin C. Loadings were equivalent to $50\mu\text{g}$ (lanes 1 and 3) and $100\mu\text{g}$ (lanes 2 and 4) dry weight of cells.

5.6.2 Analysis of LPS fatty acid composition.

Whole cells of *P. aeruginosa* PAO1 were assayed for fatty acids after acid hydrolysis. Although this method degrades the cyclopropane acids (17: O_{cyc} and 19: O_{cyc}) and can produce a number of artefacts in the chromatograms (Wilkinson, 1974), the amide-linked hydroxy fatty acid (3OH-C₁₂: O) is released more effectively by acid hydrolysis (Lambert and Moss, 1983). Most other fatty acids are ester-linked to the glucosamine backbone of lipid A in LPS (Wilkinson, 1983).

Early stationary phase whole cells grown under iron-plentiful and iron-depleted conditions in the presence and absence of sub-MICs of mitomycin C were analysed following acid hydrolysis (section 3.4.5). The results of the GLC analysis of the fatty acids of LPS from PAO1 are shown in table 5.6.2 (section 3.4.6).

Table 5.6.2 Fatty acid hydrolysis of *P. aeruginosa* PAO1 whole cells cultivated in CDM+Fe and CDM-Fe.

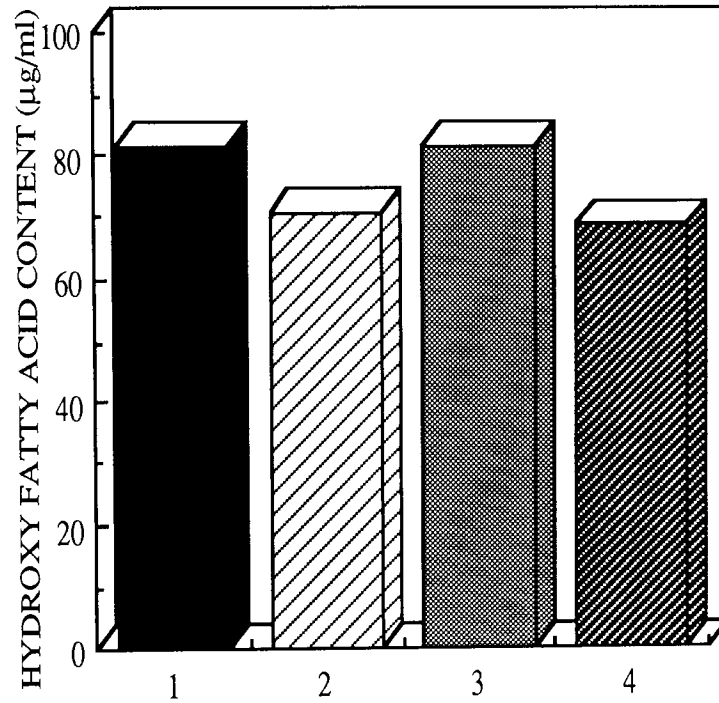
Fatty acid	CDM+Fe ($\mu\text{g.ml}^{-1}$ cells)	CDM+Fe with Mitomycin C ($\mu\text{g.ml}^{-1}$ cells)	CDM-Fe ($\mu\text{g.ml}^{-1}$ cells)	CDM-Fe with Mitomycin C ($\mu\text{g.ml}^{-1}$ cells)
11:0	19.6	13.3	19.6	14.4
3OH 10:0	15.5	12.2	16.7	12.0
12:0	3.2	0.41	1.8	0.42
13:0	4.4	30.7	2.2	10.8
2OH 12:0	24.2	21.4	28.5	21.7
3OH 12:0	42.0	37.1	36.4	34.9
14:0	3.9	4.2	4.9	4.2
16:1	16.7	19.7	26.1	47.5
16:0	119.1	125.8	148.5	96.2
17:0 _{cyc}	80.0	80.0	80.0	80.0
18:1	210.7	195.4	207.3	206.1
18:0	5.0	0.35	5.2	3.4

Fatty acids were identified on the basis of their retention times compared to standards (bacterial acid methyl ester mix 10mg.ml⁻¹). Integration readings were calculated for each peak and quantified by comparison with the value of the internal standard (C_{17:0} heptadecanoic acid). The fatty acid composition of LPS extracted from CDM+Fe and CDM-Fe cultures were similar, except there was more C_{16:0} present in LPS from cells grown under iron-depletion. C_{16:0} is a major component of whole cell phospholipids (Hancock and Meadow, 1969). The increased level of C_{16:0} in LPS from CDM-Fe cultures may be due to the presence of some phospholipids in the extracted material. The fatty acid composition was similar to that found by other workers (Hancock and Meadow, 1969; Ikemoto *et al.*, 1978; Lambert and Moss, 1983). The predominant fatty acids include C_{16:0} and C_{18:1}, with a smaller proportion of C_{11:0}, 3OH-C_{10:0}, 2OH-C_{12:0} and 3OH-C_{12:0}. Minor and probably insignificant differences between the relative proportions of certain fatty acids in mitomycin C treated cells and the untreated controls were observed. A 10% reduction in the hydroxy fatty acids was found for PAO1 grown in the presence of sub-MICs of mitomycin C under both iron-plentiful and iron-depleted conditions, however, this was not considered to be significant (Fig 5.6.2). Therefore no further experiments were carried out.

5.6.3 2-Keto 3-deoxy-D-manno-2-octulosonic acid (KDO) content of LPS from *P. aeruginosa*.

The KDO content was determined for *P. aeruginosa* grown in iron-plentiful (Fig 5.6.3.1) or iron-depleted conditions (Fig 5.6.3.2), in the presence and absence of sub-MICs of mitomycin C. Shown are strains PAO1, 6750, M7 and AK1012. Values were the mean of three determinations per sample and these were standardised by obtaining a ratio to the protein content (µg of KDO per mg of protein; see section 3.4.8). The ratios obtained for the mitomycin C treated cells of PAO1, 6750 and M7 are significantly lower, decreasing by up to 4-fold, indicating perturbed expression of LPS. However, strain AK1012 displayed a comparatively low KDO value, which appeared to be unaffected by mitomycin C. The KDO content appeared to be unaffected by the presence of iron, although the treated PAO1 cells was noted to have a much higher KDO content than its iron-depleted counterpart. The reason for this is unclear, although interference by other bacterial cell components may have affected these results.

Fig 5.6.2



The effect of sub-MICs of mitomycin C on the hydroxy fatty acid content of LPS of *P. aeruginosa* PAO1 in iron-plentiful (lanes 1 and 2) and iron-depleted (lanes 3 and 4) CDM batch culture and with $0.05\mu\text{g}\cdot\text{ml}^{-1}$ mitomycin C (lane 2 and 4).

Fig 5.6.3.1

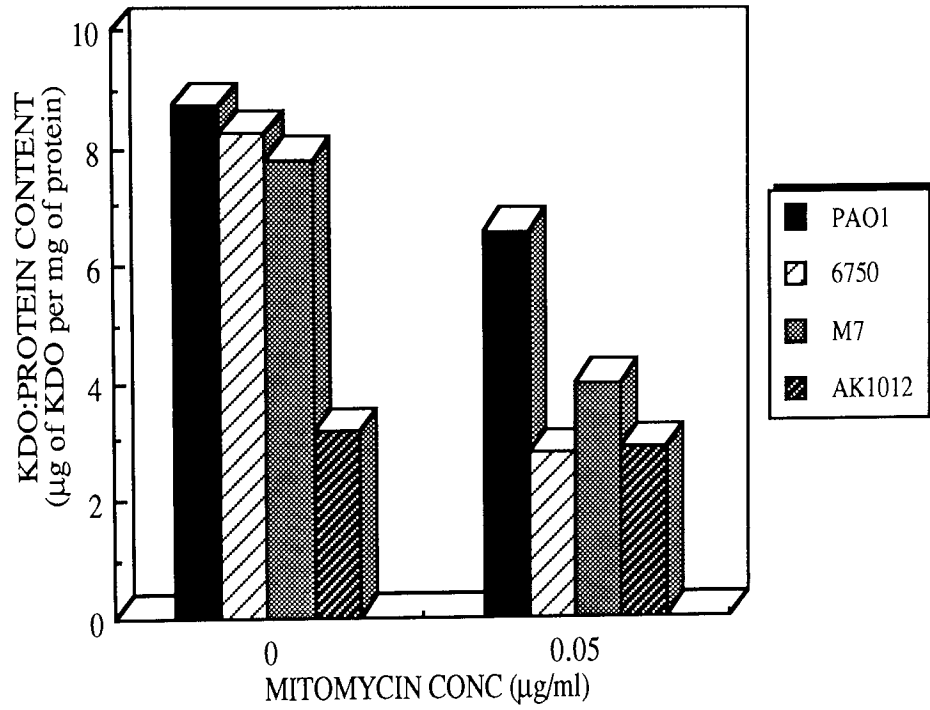
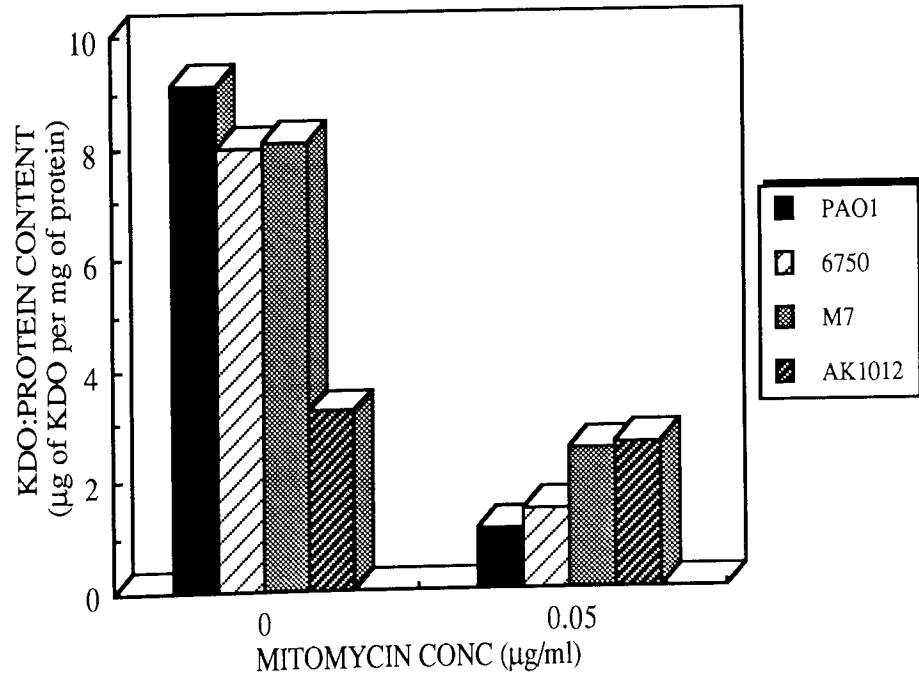


Fig 5.6.3.2



The effect of sub-MICs of mitomycin C ($0.05\mu\text{g}\cdot\text{ml}^{-1}$) on the KDO content of LPS of *P. aeruginosa* strains PAO1, 6750, M7 and AK1012, in iron-plentiful (Fig 5.6.3.1) and iron-depleted (Fig 5.6.3.2) CDM batch culture.

5.6.4 The effect of mitomycin C on the hexose content of LPS of *P. aeruginosa*.

The O-antigen of *P. aeruginosa* is rich in amino sugars (Kropinski *et al.*, 1982; 1985; Wilkinson, 1983). The KDO assay measures residues in the core region of LPS (Kropinski *et al.*, 1982), whereas the assay for amino sugars detects residues both in the core-lipid A region and O-polysaccharide (Wilkinson, 1983).

A quantitative sugar analysis was conducted using the neutral sugar rhamnose as a standard (see section 3.4.9). *P. aeruginosa* strains PAO1, 6750, M7 and AK1012 were grown in the presence and absence of sub-MICs of mitomycin C to early stationary phase under high (Fig 5.6.4.1) and low iron conditions (Fig 5.6.4.2). The values represent the mean of 3 determinations per sample and these were standardised by obtaining a ratio to the protein content (μg of rhamnose per mg of protein). It can be noted that the hexose: protein ratio in the mitomycin C treated cells is significantly lower for strains PAO1, 6750 and M7, decreasing by up to 4-fold. Mitomycin C had little effect on AK1012 which expressed a comparatively low hexose content in the untreated cells, as expected. The ratios obtained for strains PAO1, 6750 and M7 display an increased value for cells grown in the absence of iron, although the significance of this is not clear.

Fig 5.6.4.1

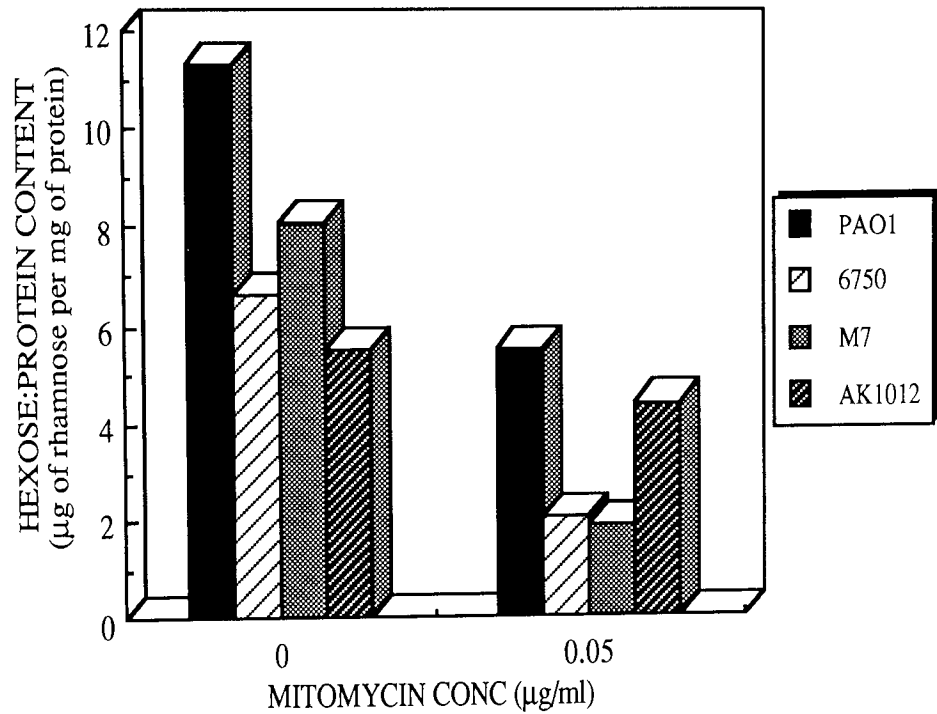
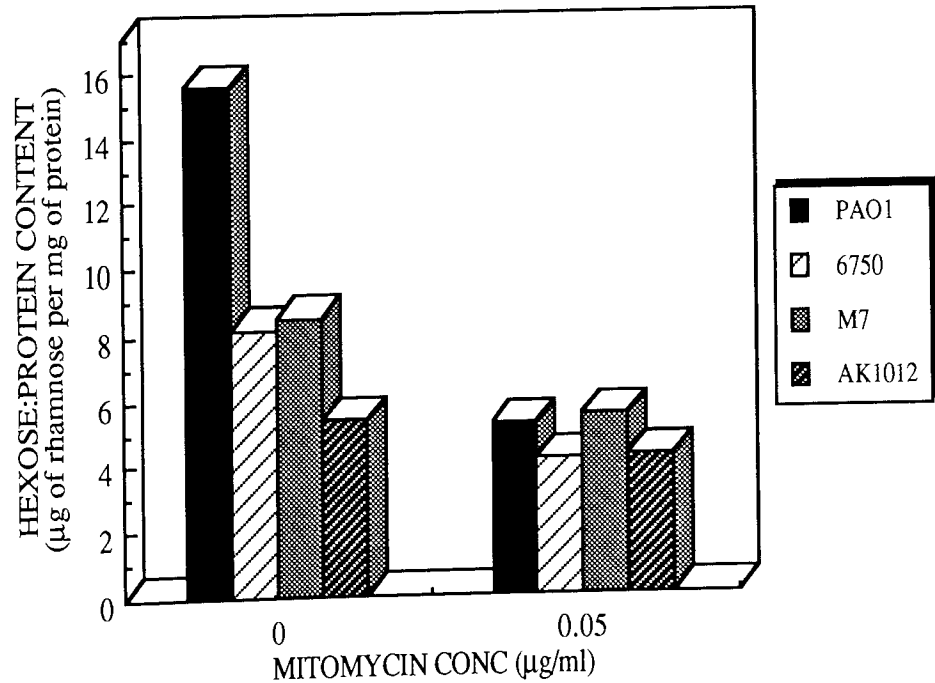


Fig 5.6.4.2



The effect of sub-MICs of mitomycin C ($0.05\mu\text{g}\cdot\text{ml}^{-1}$) on the hexose content of LPS of *P. aeruginosa* strains PAO1, 6750, M7 and AK1012, in iron-plentiful (Fig 5.6.4.1) and iron-depleted (Fig 5.6.4.2) CDM batch culture.

5.7 DISCUSSION.

Increased susceptibility to microbial infection in patients on chemotherapeutic and immunosuppressive drug regimens is a well known and constant factor in cancer patient management (Goldschmidt and Bodey, 1972; Moody *et al.*, 1978). *P. aeruginosa* is frequently responsible for infections in cancer patients, in particular leukaemic patients following chemotherapy (Richet *et al.*, 1989). The anti-tumour agent mitomycin C has been shown to exhibit antimicrobial activity against Gram-positive and Gram-negative bacteria, including *P. aeruginosa* (Hamilton-Miller, 1984). However, little work has been done to define optimum conditions for combined antineoplastic and antibacterial agents in inhibiting bacterial growth (Ghannoum *et al.*, 1989).

The surface properties and composition of bacteria are known to play an important role in infection (Costerton, 1979; Brown and Williams, 1985b; Smith, 1990). Studies have shown that sub-MICs of certain antibiotics can alter the morphology and virulence of bacterial species, as well as their susceptibility to the human host immune system (Ahlstedt, 1981; Lorian and Atkinson, 1984; Brown and Williams, 1985b; Kadurugamuwa *et al.*, 1985a; 1985c; Lorian and Ernst, 1987). Despite the known effects of iron depletion and sub-MICs of antibiotics, few workers have taken these factors into account in their *in vitro* studies. In this study the effects of sub-MICs of mitomycin C on several strains of *P. aeruginosa* under iron-plentiful and iron-depleted conditions was investigated.

Growth of *P. aeruginosa* in the presence of mitomycin C, showed OprG to be present only under iron-plentiful conditions (see section 4.3). Its expression did not appear to be influenced by mitomycin C. An additional protein band (molecular weight 32 kDa) above OprG was observed in the OM preparation of strains 6750 and M7 treated with mitomycin C regardless of media iron concentration. The role played by this protein is unclear. However, Cochrane (1987) noted that sera from patients whose lungs were colonised with mucoid *P. aeruginosa* contained an additional antigen of molecular weight 32 kDa, which was immunogenic. The antigenicity of the OM components and in particular the 32 kDa protein of *P. aeruginosa* 6750 was investigated by immunoblotting with serum from a leukaemic patient. The serum was absorbed with extracted LPS from 6750 prior to

immunoblotting so that the response obtained represented an antibody response to OMPs and not against co-migrating LPS (Poxton *et al.*, 1985). Interestingly, these preliminary data indicate that *P. aeruginosa* grew under iron-plentiful conditions in the leukaemic patient, as judged by the apparent lack of recognition of IRMPs under low iron conditions. This is in contrast to studies with bacteria taken directly from human infections without subculture (Brown *et al.*, 1984; Shand *et al.*, 1985; Ward *et al.*, 1988), and in experimentally infected animals (Griffiths *et al.*, 1983; Cochrane *et al.*, 1987), which show IRMPs to be expressed in OM of *P. aeruginosa*. The 32 kDa protein and OprE induced in the presence of mitomycin C were only faintly detected, indicating their lack of importance as targets of host defences *in vivo* in leukaemic patients. Furthermore, immunoblotting demonstrated strong recognition of OprF, OprL and protein I (OprI) by serum antibodies. The humoral response to *P. aeruginosa* OMPs has been investigated following subcutaneous infection in mice (Hedstrom *et al.*, 1984). The results showed production of antibodies to OprF, OprL and OprI. Sera from cystic fibrosis patients with *P. aeruginosa* lung infection have also been used in immunoblotting studies (Anwar *et al.*, 1984). Such sera detected IRMPs, protein D and OprL and showed a strong reaction to OprF and OprG. Recognition of OprG was not observed in the work of this thesis. This probably reflects a difference in immunological recognition of the OM antigens of clinically isolated bacteria from the patient and that of laboratory based bacteria grown in *in vitro* conditions. This effect has been observed previously (Kadurugamuwa, 1985). In addition *P. aeruginosa* OMPs used in this study were treated with SDS and 2-mercaptoethanol. Conformational epitopes in the antigens may have been affected by this treatment, which could explain the lack of recognition of OprG (Mutharia and Hancock, 1985).

LeVatte and Sokol (1989) showed that growth of *P. aeruginosa* in medium containing sub-MICs of antibiotics repressed surface expression of the ferripyochelin binding protein (FBP) measured by ¹²⁵I labelling. They suggested that exposure to sublethal doses of tobramycin, tetracycline or chloramphenicol can alter the ability of these organisms to acquire iron. However, this effect is not due simply to the presence of any antibiotic in the medium since ciprofloxacin was found not to repress expression of FBP (LeVatte and Sokol, 1989) or affect secretion of siderophores (Morris and Brown, 1988). This repression was observed with antibiotics which affected protein synthesis (Martin and

Beveridge, 1986; Davis, 1987; LeVatte and Sokol, 1989), rather than ciprofloxacin, where the primary action is on DNA gyrase (Gellert, 1981). If iron transport systems can be repressed or circumvented, the bacteria should be less virulent and this may have important implications in antimicrobial therapy. Thus, even if antibiotics do not achieve sufficient concentrations in tissues or serum to kill or clear the organisms, they may still be effective in treatment of infections as a consequence of their repression of surface components for adherence or iron acquisition (LeVatte and Sokol, 1989).

Surface hydrophobicity plays a significant role in the engulfment of bacterial cells by polymorphonuclear leukocytes (PMNs; Van Oss, 1978; Stendahl, 1983). Phagocytic cells such as PMNs appear to recognise foreign particles by at least two mechanisms (Van Oss, 1978; Stendahl, 1983). The first is mediated via specific ligand-receptor interactions. Such ligands include the Fc portion of IgG and the C3b component of complement. However, general physicochemical properties such as surface hydrophobicity and surface charge also play a role, and IgG and C3b are known to increase surface hydrophobicity of opsonised micro-organisms (Van Oss, 1978; Stendahl, 1983). In bacterial infection, hydrophobicity measurements are therefore a nonspecific method of assessing changes in cell-surface properties (Absolom, 1988). Such changes in surface hydrophobicity might result from compositional and/or structural changes in more than one surface component (Allison *et al.*, 1990).

The cell surface of *P. aeruginosa* grown in the presence of sub-MICs of mitomycin C were markedly more hydrophobic than cells grown without the antibiotic. This effect was more pronounced under iron-depletion. Previous studies have shown that surface hydrophobicity of *K. pneumoniae* is increased when bacterial cells are grown in the presence of cephalosporins (Kadurugamuwa *et al.*, 1985a) and for *P. aeruginosa* in 6-aminopenicillanic acid (Godfrey and Bryan, 1989). In addition, these workers obtained preliminary evidence that filamented bacteria, resulting from, β -lactam action, are more liable to phagocytosis than non filamented cells by rabbit PMNs (Kadurugamuwa *et al.*, 1985b). Similar observations have been reported by other investigators (Lorian and Atkinson, 1984).

Cells grown under different nutrient depletions might have altered surface antigens which would be opsonised to different extents (Anwar *et al.*, 1983a). Envelope components which influence bacterial surface properties of Gram-negative bacteria include, OMPs and the heterogeneity of the LPS occurring as a result of phenotypic variation (Goldman and Leive, 1980; Palva and Makela, 1980; Williams *et al.*, 1986). The complete O-antigen of LPS confers hydrophilic characteristics upon bacterial cell surfaces (Williams *et al.*, 1988). Therefore, changes in surface hydrophobicity could result from mitomycin C reducing or preventing the production of LPS. *In vivo* such effects might reduce the capacity of a bacterium to survive in the host. Many studies have shown that antibiotics, in addition to their effects on primary targets such as protein synthesis or DNA supercoiling, have multiple secondary effects on the bacterial cell, especially on its envelope. Taylor *et al.*, (1982) described the influence of amdinocillin (mecillinam) on the length of the LPS chain and together with Kadurugamuwa *et al.*, (1985a) observed an influence of antibiotics on the production of capsular polysaccharides in *E. coli* and *P. aeruginosa*.

Different authors have indicated that such influences on the cell surface are closely related to the influence of antibiotics on various host-parasite interaction processes, such as adherence, phagocytosis, serum resistance or immune response to OM components (Ofek *et al.*, 1979; Taylor *et al.*, 1982; Bassaris *et al.*, 1984; Leying *et al.*, 1986). LPS is an important virulence factor of *P. aeruginosa* and the structure of the O-antigen polysaccharide chain plays a significant role in the ability of the bacterium to evade host defence mechanisms (Cryz *et al.*, 1984; Engels *et al.*, 1985; McCallum *et al.*, 1989). The LPS (Wilkinson and Galbraith, 1975; Wilkinson, 1983) and fatty acid content of *P. aeruginosa* (Lambert and Moss, 1983; Ikemoto *et al.*, 1978) have been investigated previously. However, these studies did not address the phenotypic variability of the bacterial cell. Growth rate, specific nutrient limitation, temperature and antibiotics have been shown to alter the phospholipid, fatty acid and LPS content of *P. aeruginosa* (Gilbert and Brown, 1978; Kropinski *et al.*, 1982, 1987; Conrad and Galanos, 1989). The purpose of these experiments was to investigate, both qualitatively and quantitatively, the effect of sub-MICs of mitomycin C and iron-depletion on the LPS profile (quantified by changes in KDO and hexose content) and the cellular fatty acid content of *P. aeruginosa* grown in batch culture.

The silver-stained SDS-polyacrylamide gels of proteinase K digests of whole cells of *P. aeruginosa* strains PAO1, 6750, M7 and PAJ revealed great diversity in the pattern of O-polysaccharide side chains, whereas the rough strain AK1012, as expected, appeared to be devoid of O-antigen material. The LPS formed a ladder-like pattern in which each band represents the rough core of LPS substituted with a different number of O-antigen repeat units (Goldman and Leive, 1980). It is well known that the molecular weight of LPS is heterogeneous and attributed to the chain length of its polysaccharide moiety (Chester and Meadow, 1975; Jann *et al.*, 1975). However, it is not clear whether both smooth and rough types of LPS are expressed simultaneously on the surface of a single cell, or cells possessing smooth and rough LPS coexist as heterogeneous cell populations in cultures of a single strain (Yokota *et al.*, 1989). The profiles of strains PAO1, 6750, M7 and PAJ showed a significant increase in the lipid A-core region and low molecular weight material which was more densely stained in the presence of mitomycin C, compared to the LPS from untreated cells. However, no visible alteration in the LPS profile was observed for strain AK1012. The physical state of the core and of the O-polysaccharide side chain has been found to influence virulence greatly, with strains possessing complete core and side chain being far more virulent than those with an abbreviated structure (Cryz *et al.*, 1984; Engels *et al.*, 1985). This was thought to allow the strains to escape from normal host defence systems of which phagocytosis appears to be the most critical for *P. aeruginosa* (Young, 1980). The authors suggested that the LPS O-side chain polysaccharide acts to mask the complement binding site that initiates cell lysis. This site is thought to be represented by the core-lipid A region of LPS or a membrane protein or both (Cryz *et al.*, 1984).

Low-level impermeability-type aminoglycoside resistance in *P. aeruginosa* has been shown to result from conversion of smooth LPS to superficial or deeper rough LPS phenotypes (Bryan *et al.*, 1984). The authors suggest that the repeating side chains of LPS are necessary either for direct binding of the aminoglycoside or for providing a suitable radius of hydrophilicity in relationship to OM pores to enhance access of aminoglycosides to that route through the OM. The marked reduction of repeating side chain sugars could contribute to a reduction in the hydrophilic nature of a bacterium (Bryan *et al.*, 1984). In this study there was no observable decrease in the LPS O-antigen repeat units for strains

PAO1, 6750, M7 and PAJ. However, the significant increase in the cell surface hydrophobicity, together with a marked reduction in KDO and hexose content, suggests that these strains possess an LPS lacking at least a portion of the repeating O-polysaccharide side chain.

Immunoblotting results indicated that serum contained antibodies which reacted mainly with high-molecular-weight LPS from *P. aeruginosa* PAO1. Similar results have been observed by Cochrane (1987). The presence of sub-MIC of mitomycin C did not appear to affect the antigenic determinants present in LPS since IgG antibodies in the serum reacted with the LPS regardless of the culture conditions.

The hydroxy fatty acids, which are unique to the lipid A component of Gram-negative bacteria, were used as an independent measure of LPS. GLC analysis indicated that LPS prepared from PAO1 contained all the fatty acids which are commonly present in strains of *P. aeruginosa* namely, 3OH-C_{10:0}, C_{12:0}, 2OH-C_{12:0}, 3OH-C_{12:0} and C_{16:0} (Wilkinson and Galbraith, 1975; Kropinski *et al.*, 1982; Wilkinson, 1983). Similar constituents and relative concentrations found in this study have been described by other researchers (Drewry *et al.*, 1973; Wilkinson and Galbraith, 1975; Kropinski *et al.*, 1985). No major differences were found between the fatty acid composition of LPS from iron-plentiful and iron-depleted early stationary phase cultures, which has also been observed previously (Cochrane, 1987; Ward, 1987). In the presence of sub-MICs of mitomycin C the hydroxy fatty acids decreased by 10%, which was not considered to be significant. However, together with the other assays performed, namely, hydrophobicity, KDO and carbohydrate, these would indicate that mitomycin C is associated with a decrease in the LPS content of the OM of *P. aeruginosa* strains PAO1, 6750 and M7. Recently, it has been proposed that many serotypes of *P. aeruginosa* produce two chemically and antigenically distinct LPS molecules, one of which is a common antigen with a short-chain polysaccharide possessing a unique core-lipid A structure and the other a longer high-molecular weight B-band LPS (Rivera *et al.*, 1988; Rivera and McGroarty, 1989). The authors suggest that for strains containing both A- and B-band type molecules, the longer B-band polymers extend from the surface and constitute the main antigenic structure exposed on the cell. The B bands have been found to be relatively low in rhamnose compared to the A bands (Rivera and

McGroarty, 1989). Since a significant decrease in the hexose content of mitomycin C-treated cells is observed, perhaps this antibiotic exerts its effect on the shorter A-band type molecules, which may be covered and masked by the B-band O-polymers (Rivera and McGroarty, 1989) and therefore accounting for no observable differences found in the O-antigen material on the LPS gels. In addition, since the B-band type molecules are thought to represent the main antigenic determinant on the cell surface this may explain the apparent lack of effect of mitomycin C in the immunoblotting studies. Lam *et al.*, (1989) have suggested that A-band LPS may be the major LPS antigen in non-typable clinical isolates and a common antigen among other *P. aeruginosa* strains, while B-band LPS is responsible for the serotype specificity of an organism and therefore each serotype has a unique type of B-band LPS on its surface. However, under certain conditions, such as prolonged antibiotic therapy, clinical isolates are found to be non-typable and appear to lose the O-polymer-containing B bands (Hancock *et al.*, 1983; Bryan *et al.*, 1984; Godfrey *et al.*, 1984). For such clinical isolates the A bands may become exposed and serve as an important antigenic determinant. However, since mitomycin C appeared to have very little effect on the rough strain AK1012 and the MIC is the same as the other *P. aeruginosa* strains used in this study, other alterations in the OM may negate the effect of phenotypically induced changes in *P. aeruginosa* LPS of strains PAO1, 6750, M7 and PAJ.

The results of this study indicated that antibacterial agents, even at concentrations below their MIC, have pronounced effects on the surface properties and quantitative composition of the OM of bacteria. The link between the primary mechanism of action and these secondary effects is unknown. A major goal of studies of combined-drug therapy for bacteria-infected leukaemic patients is to define mixtures of drugs which mutually potentiate inhibitory effects on bacterial growth so that minimal levels of toxic drugs may be used. This must include not only studies with mixtures of antibacterial drugs, but also evaluations of the antibacterial effects of the antineoplastic drugs and of combinations of antibacterial plus antineoplastic agents.

RESULTS CHAPTER 6

6. THE ENVIRONMENT OF THE LEUKAEMIC PATIENT.

6.1 INTRODUCTION.

Serum iron plays an important role in the body's defence against many bacterial infections (Sussman, 1974; Weinberg, 1974; 1978; Bullen, 1981; Griffiths, 1983b; 1987a). The response of the human body to infection generally results in a reduction in the plasma concentration of iron. This decrease is thought to be attributable primarily to increased storage of iron in the liver (Kluger and Bullen, 1987) and within cells of the reticuloendothelial system (Lee, 1983). To a lesser extent, iron absorption through the intestine, which is normally the major source of body iron, is reduced (Flanagan, 1989). This results in the hypoferraemia of infection and is an adaptive host defence response. By decreasing the availability of iron to the infecting organism the host might be attenuating microbial growth. Several lines of biological evidence suggest that an excess of body iron stores is frequently associated with an enhanced incidence of malignancy (Weinberg, 1983; Stevens *et al.*, 1986; 1988; Good *et al.*, 1988). Infectious microorganisms and neoplastic cells require iron for growth and are specifically adapted to compete with host cells for iron (Weinberg, 1984). This situation is probably best exemplified in individuals with idiopathic haemochromatosis, a genetic disease characterised by iron overload and is associated with a high risk of liver cancer (Bergeron *et al.*, 1985; Weinberg, 1986; Grootveld *et al.*, 1989; Harju, 1990). Another case is the excessive oral ingestion of iron, in the form of home-brewed spirits in South African gold miners, which has been associated with an extraordinarily high incidence of primary hepatoma (Bothwell *et al.*, 1964; Robertson *et al.*, 1971; Weinberg, 1983). Furthermore, iron-dextran complex injections have been connected with the development of sarcoma at the injection site in animals and humans (Robertson and Dick, 1977; Weinberg, 1981). Excessive body iron stores and inappropriate iron administration may therefore interfere with natural body defences and promote cancer, while relatively low iron stores may be protective against cancer (Weinberg, 1981; Hann *et al.*, 1988; Selby and Friedman, 1988; Stevens *et al.*, 1988).

The rationale for this study was based on three observations which are well established in the literature but seldom have been linked together and studied in a coordinated fashion. First, iron in the circulation in excess of that required to saturate available transferrin is associated with increased susceptibility to infection (Murray *et al.*, 1978; Weinberg, 1978; Bullen, 1981; Hunter *et al.*, 1984b; Bullen *et al.*, 1991). Second, many patients with acute leukaemia have elevated serum iron and abnormalities in iron-binding proteins (Caroline *et al.*, 1969; Hunter *et al.*, 1984b). Finally, patients with leukaemia are highly susceptible to bacterial infections (Bodey, 1977; 1986).

Therefore, the present investigation was motivated by the consideration that a host environment rich in iron represented by high serum iron and high transferrin saturation, with low serum transferrin and total iron binding capacity (TIBC) might offer more favourable growth conditions to invading bacteria in the leukaemic host. The study, in collaboration with Dr. N. Patton, was divided into three phases:

Phase 1: The serum iron, transferrin level/saturation and TIBC status of twenty randomly selected, newly diagnosed leukaemic patients to be determined.

Phase 2: From newly diagnosed patients with leukaemia or on admission to hospital, a blood sample sufficient to produce approximately 3-4ml serum to be taken. These samples would be stored at Aston University and used to determine the pre-infection immune and iron status if the patient proceeds to phase 3.

Phase 3: Any leukaemic patient (including those not in phase 2) who develops a suspected or confirmed gram-negative septicaemia:

- 1) A 3-4ml serum sample at suspicion/diagnosis of infection and then at frequent intervals (4-5 days), and a sample one month after recovery (if possible) to be taken.
- 2) A 1ml serum sample (from above) to be analysed as in phase 1.
- 3) The causative organism where isolated to be obtained from the microbiology department.
- 4) Details of each patient to accompany each sample.

6.2 IRON STATUS OF NEWLY DIAGNOSED LEUKAEMIC PATIENTS.

Sera from 20 randomly-selected, newly-diagnosed leukaemic patients were obtained. The results of assays for serum iron, transferrin, percentage saturation and TIBC are shown in table 6.2.1 and 6.2.2. Only 7 patients (35%) had all the values in the normal range. Eleven of the 20 patients (55%) had abnormally elevated percentage saturation of transferrin with iron. This was due either to elevated serum iron levels, decreased transferrin levels or both.

6.3 ASSESSMENT OF IRON STATUS DURING INFECTION.

Serial measurements of iron-related parameters were performed to explore the hypothesis that an inability to reduce the saturation of serum transferrin with iron contributes to susceptibility to infection in patients with leukaemia. With onset of fever or the establishment of infection, the generalised response is for the serum iron level to fall markedly, while plasma transferrin level increases resulting in a reduction in the percentage saturation of transferrin with iron (Hunter *et al.*, 1984b; Ward, 1986).

Plasma TIBC, which measures the ability of plasma proteins, particularly transferrin to bind added iron, is another indirect assessment of body iron stores. TIBC is inversely related to both iron stores, as measured by marrow aspiration and to plasma ferritin (Lipschitz *et al.*, 1974). The plasma ferritin level is often used clinically as an indirect measurement of body iron stores. The plasma fraction of this tissue iron storage protein is directly related to body iron stores among healthy persons (Lipschitz *et al.*, 1974). However, the presence of chronic infection or malignancy increases ferritin levels independently of body iron stores (Finch *et al.*, 1986). Therefore, an accurate assessment of the predictive ability of the plasma ferritin level as the measurement of iron stores in a population requires a long interval between measurement and cancer incidence (Selby and Friedman, 1988). Consequently, plasma ferritin levels were not assessed in this study.

In this study the iron status of 9 patients, 3 *P. aeruginosa*, 6 *E. coli* and 2 *K. pneumoniae* throughout an infection episode were assessed. The same patient developed an *E. coli* followed by a *P. aeruginosa* infection and another patient developed 2 *E. coli* septicaemias.

Table 6.2.1 Assessment of iron status of 20 newly diagnosed leukaemic patients I.

	NAME	SEX	DATE	TOTAL IRON IN SERUM ($\mu\text{mol.L}^{-1}$)	TRANSFERRIN LEVEL (g.L^{-1})	TIBC
1	A.P	F	3.2.89	*31.3	*1.64	41
2	H.H	F	31.1.89	*36.5	2.00	50
3	P.P	F	1.2.89	*36.5	3.10	77.5
4	S.F	F	7.2.89	*37.4	2.39	59.8
5	A.F	F	7.2.89	24.9	2.39	59.8
6	H.W	F	14.4.89	*36.2	*1.78	44.5
7	D.T	M	7.2.89	22.4	2.15	53.8
8	R.R	M	7.2.89	*36.9	2.48	62
9	D.H	M	2.2.89	*38.9	2.14	53.5
10	W.S	M	31.1.89	25.9	2.45	61.3
11	A.B	M	31.1.89	15.6	*1.91	47.8
12	P.Y	M	31.1.89	26.0	*1.43	35.8
13	D.R	M	7.2.89	29.5	*1.88	47
14	D.D	M	31.1.89	21.4	2.22	55.5
15	G.E	M	2.2.89	*41.8	2.36	59
16	J.B	M	7.2.89	18.9	2.08	52
17	D.M	M	14.2.89	19.4	3.16	79
18	M.S	M	21.2.89	*40.1	*1.73	43.3
19	M.R	M	21.2.89	24.8	2.73	68.3
20	B.G	M	13.4.89	*39.5	2.01	50.3

For iron levels- reference range: $10\text{-}32\mu\text{mol.L}^{-1}$ for males.

$5\text{-}30\mu\text{mol.L}^{-1}$ for females.

For transferrin levels- reference range: $2.0\text{-}3.6\text{g.L}^{-1}$.

TIBC (Total Iron Binding Capacity) = transferrin level X 25.

* Abnormal value.

Table 6.2.2 Assessment of iron status of 20 newly diagnosed leukaemic patients II.

	NAME	SEX	DATE	SERUM TS (%)
1	A.P	F	3.2.89	*76.3
2	H.H	F	31.1.89	+73.0
3	P.P	F	1.2.89	+47.1
4	S.F	F	7.2.89	+63.4
5	A.F	F	7.2.89	41.6
6	H.W	F	14.4.89	*81.3
7	D.T	M	7.2.89	41.6
8	R.R	M	7.2.89	+59.5
9	D.H	M	2.2.89	+72.7
10	W.S	M	31.1.89	42.3
11	A.B	M	31.1.89	+32.6
12	P.Y	M	31.1.89	+72.6
13	D.R	M	7.2.89	+62.8
14	D.D	M	31.1.89	38.6
15	G.E	M	2.2.89	+70.8
16	J.B	M	7.2.89	36.3
17	D.M	M	14.2.89	24.6
18	M.S	M	21.2.89	*92.6
19	M.R	M	21.2.89	36.3
20	B.G	M	13.4.89	+78.5

For serum transferrin saturation (TS) level- reference range: 20-50%.

% of transferrin saturation = $\frac{\text{serum iron}}{\text{TIBC}} \times 100\%$.

* abnormal serum iron + transferrin level.

+ abnormal serum iron or transferrin level.

The results of serum iron, transferrin/saturation and TIBC for leukaemic patients infected with *P. aeruginosa* are shown respectively for patients PAVI (Figs 6.3.1.1-4), PAJK (Figs 6.3.2.1-4) and PAGF (Figs 6.3.3.1-4). If the normal serum iron level is 10-32 $\mu\text{mol.L}^{-1}$ for males and 5-30 $\mu\text{mol.L}^{-1}$ for females, with the reference value for transferrin being 2-3.6g.L $^{-1}$, then all these patients have abnormal levels at onset of infection. During the initial stages of infection serum iron and transferrin levels fell, the saturation of transferrin with iron remaining above 50%, for the majority of the infection episode (reference value is 20-50%). This is probably best exemplified for PAGF (Fig 6.3.3.1-4), where the transferrin level fell during the first 2 days of infection, the serum iron level rose dramatically, and the transferrin saturation increased to 100%. The patient died of sepsis on day 4. The TIBC was generally found to decrease at onset of infection, reflecting high body iron stores, before increasing significantly. However, TIBC is influenced by a variety of chronic conditions, including infection and malignancy, generally being lower in these conditions (Selby and Friedman, 1988), therefore the significance of these results is unclear.

Similar results of iron status for *E. coli* septicaemic patients ECSM (Figs 6.3.4.1-4), ECVI (Figs 6.3.5.1-4), ECHD (Figs 6.3.6.1-4), ECFA (Figs 6.3.7.1-4), ECHH1 (Figs 6.3.8.1-4) and ECHH2 (Figs 6.3.9.1-4) are shown, respectively. Abnormal levels of serum iron, transferrin and transferrin saturation were again observed in pre-infection serum samples. The majority of patients attained 100% transferrin saturation at some point during the infection episode. The significance of TIBC is unclear, but most of the patients show an initial fall, followed by an increase on recovery.

Finally the results of the iron status for the *K. pneumoniae* septicaemic patients KPSW (Figs 6.3.10.1-4) and KPWS (Figs 6.3.11.1-4), respectively are shown. The fall in serum iron, transferrin levels and TIBC at onset of infection and the correspondingly high saturation of transferrin with iron follows a similar trend to that observed previously. All the patients appear to show an inability to reduce the percentage saturation of transferrin with iron during a febrile episode.

Fig 6.3.1.1

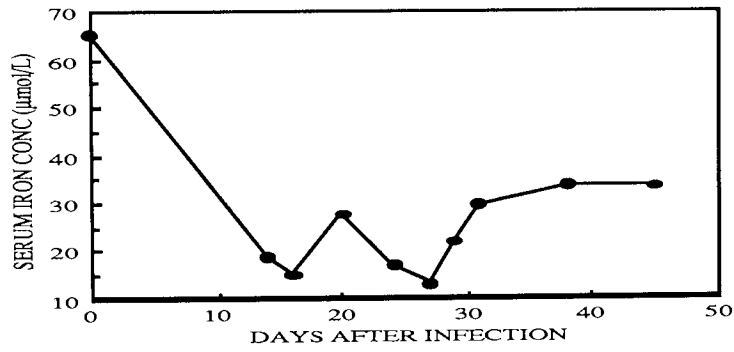


Fig 6.3.1.2

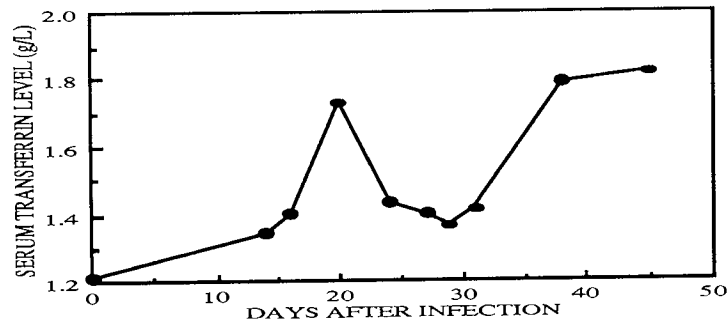


Fig 6.3.1.3

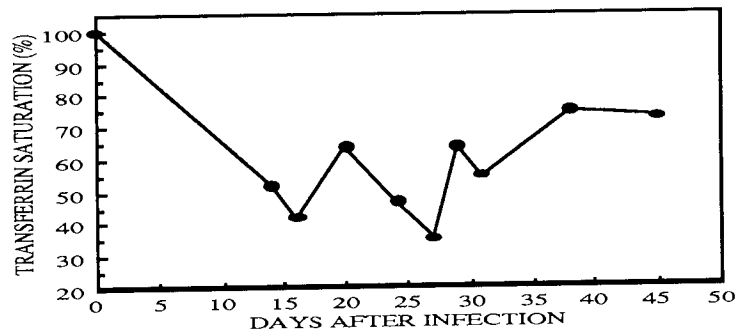
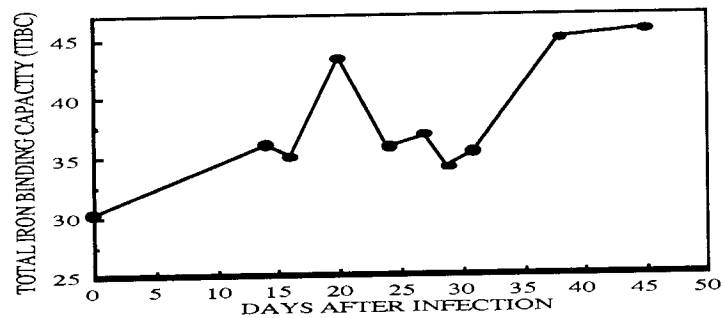


Fig 6.3.1.4



Progression of serum iron concentration (Fig 6.3.1.1), serum transferrin level (Fig 6.3.1.2), transferrin saturation (Fig 6.3.1.3) and TIBC (Fig 6.3.1.4) with infection for patient PAVI.

Fig 6.3.2.1

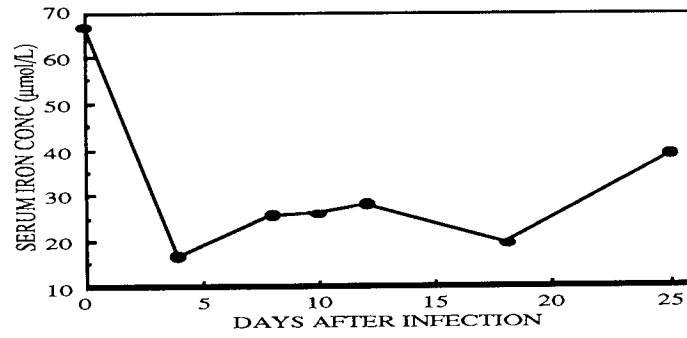


Fig 6.3.2.2

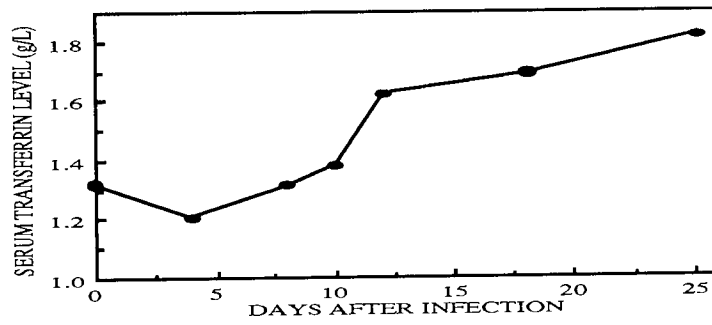


Fig 6.3.2.3

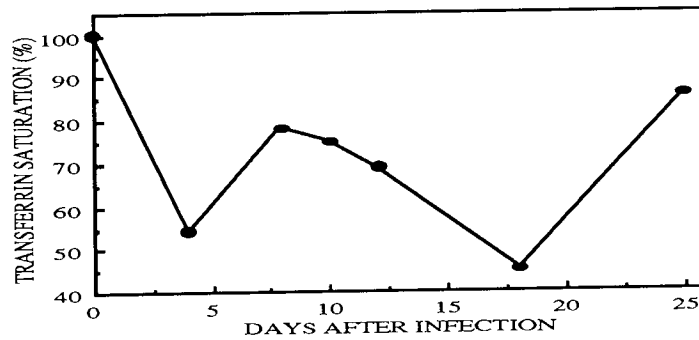
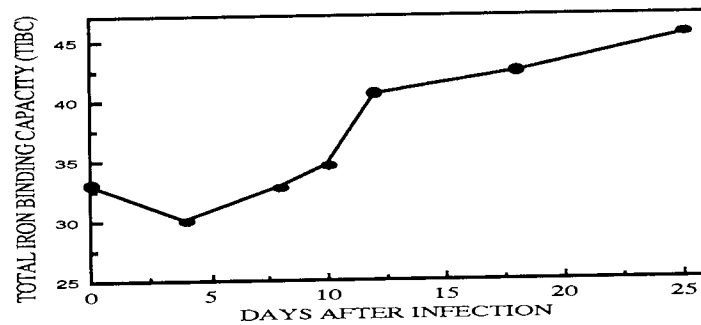


Fig 6.3.2.4



Progression of serum iron concentration (Fig 6.3.2.1), serum transferrin level (Fig 6.3.2.2), transferrin saturation (Fig 6.3.2.3) and TIBC (Fig 6.3.2.4) with infection for patient PAJK.

Fig 6.3.3.1

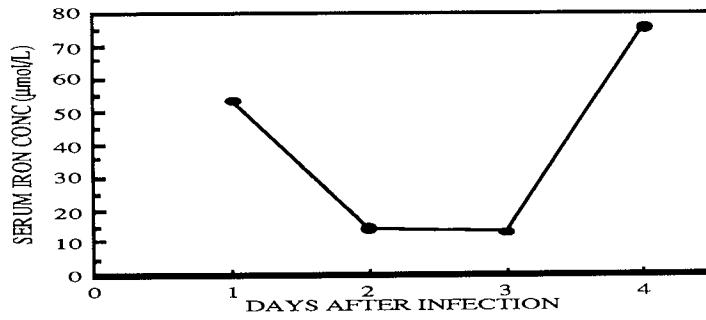


Fig 6.3.3.2

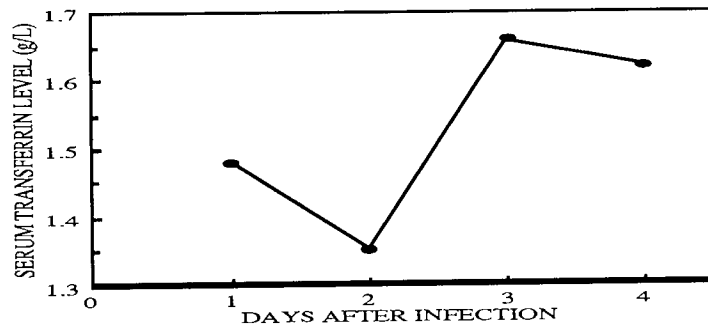


Fig 6.3.3.3

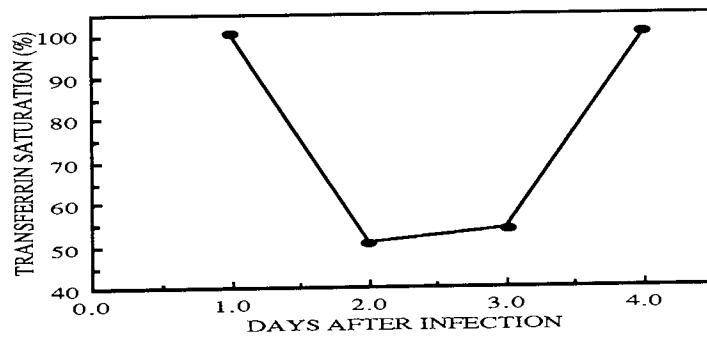
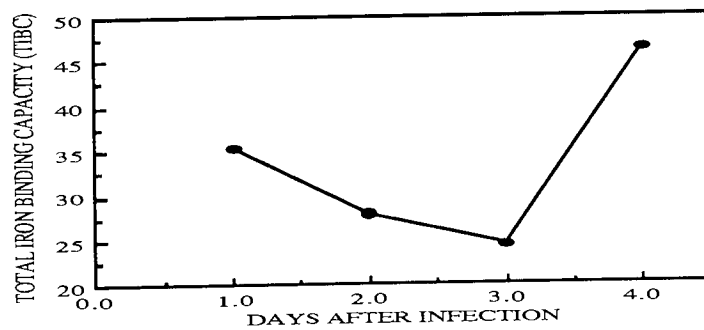


Fig 6.3.3.4



Progression of serum iron concentration (Fig 6.3.3.1), serum transferrin level (Fig 6.3.3.2), transferrin saturation (Fig 6.3.3.3) and TIBC (Fig 6.3.3.4) with infection for patient PAGF.

Fig 6.3.4.1

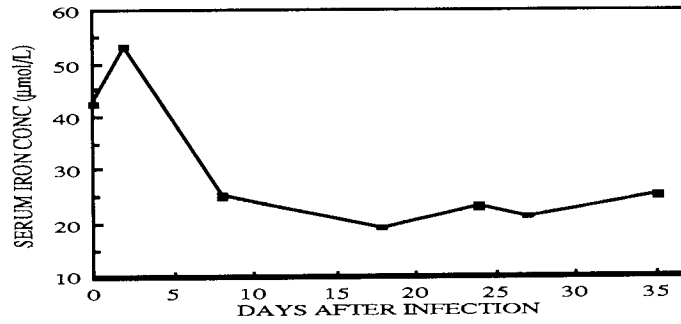


Fig 6.3.4.2

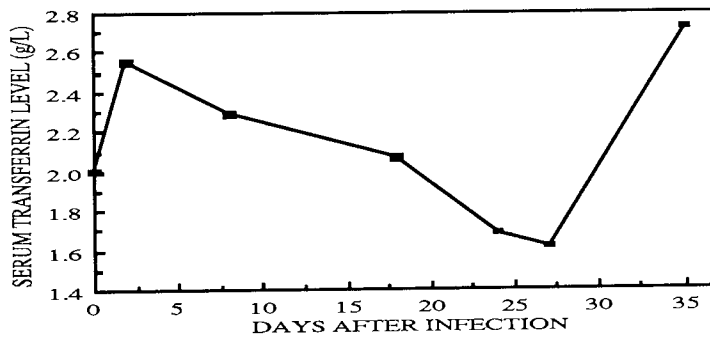


Fig 6.3.4.3

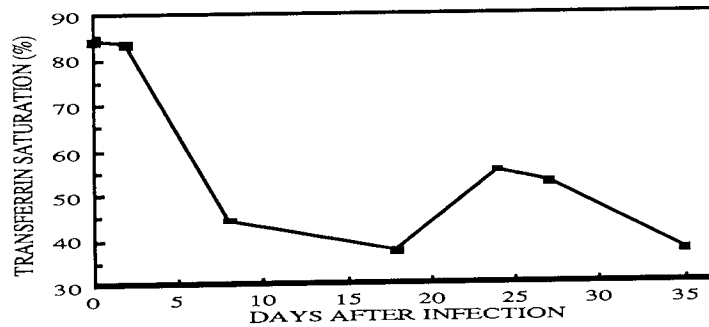
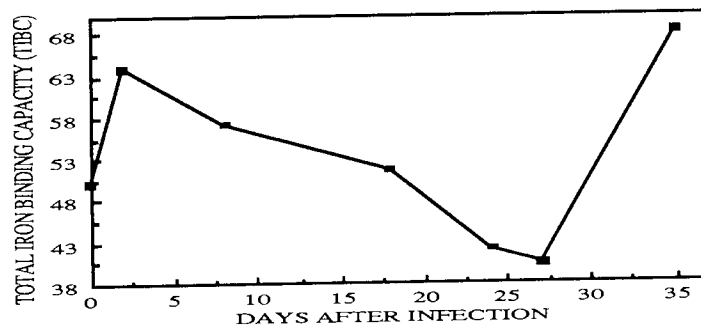


Fig 6.3.4.4



Progression of serum iron concentration (Fig 6.3.4.1), serum transferrin level (Fig 6.3.4.2), transferrin saturation (Fig 6.3.4.3) and TIBC (Fig 6.3.4.4) with infection for patient ECSM.

Fig 6.3.5.1

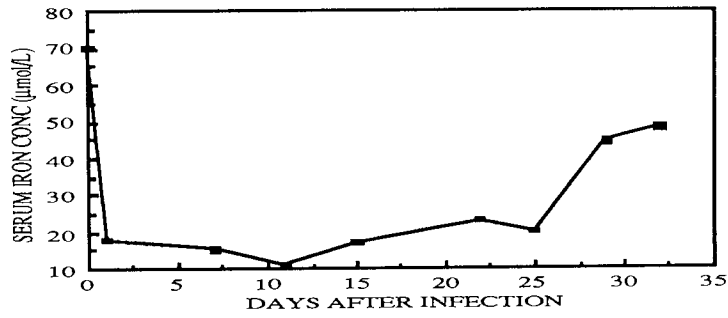


Fig 6.3.5.2

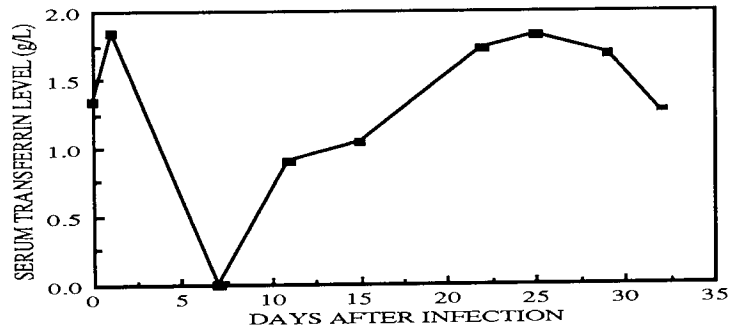


Fig 6.3.5.3

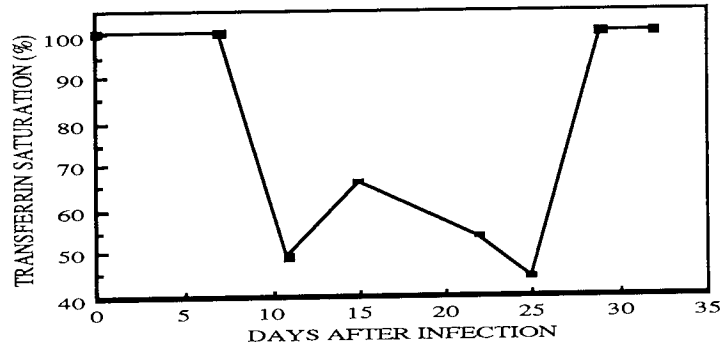
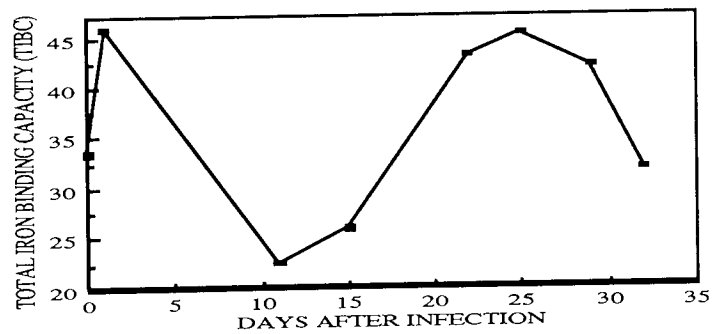


Fig 6.3.5.4



Progression of serum iron concentration (Fig 6.3.5.1), serum transferrin level (Fig 6.3.5.2), transferrin saturation (Fig 6.3.5.3) and TIBC (Fig 6.3.5.4) with infection for patient ECVI.

Fig 6.3.6.1

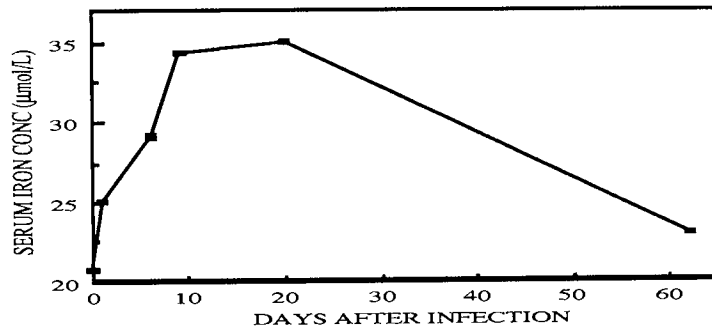


Fig 6.3.6.2

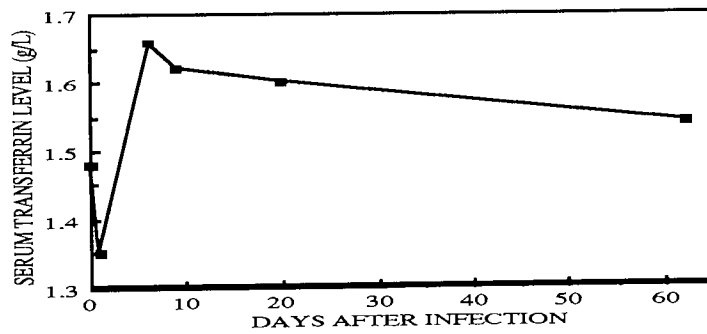


Fig 6.3.6.3

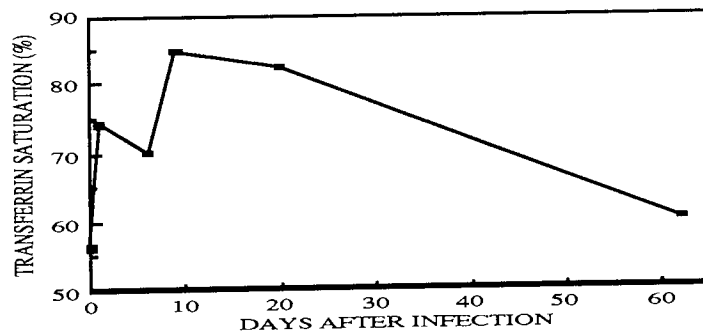
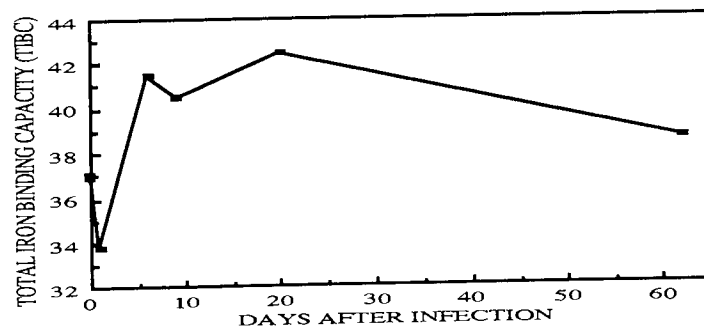


Fig 6.3.6.4



Progression of serum iron concentration (Fig 6.3.6.1), serum transferrin level (Fig 6.3.6.2), transferrin saturation (Fig 6.3.6.3) and TIBC (Fig 6.3.6.4) with infection for patient ECHD.

Fig 6.3.7.1

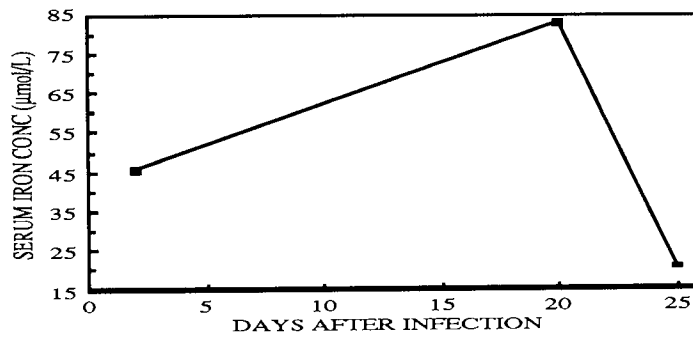


Fig 6.3.7.2

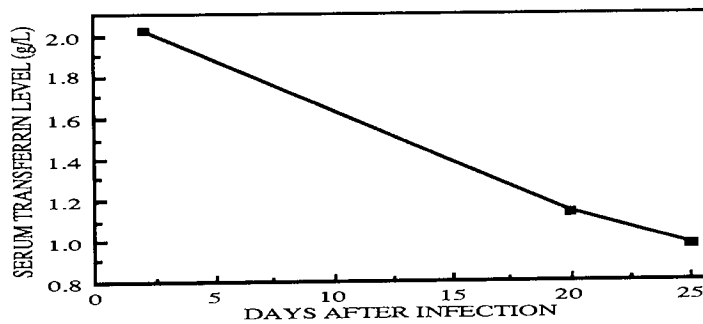


Fig 6.3.7.3

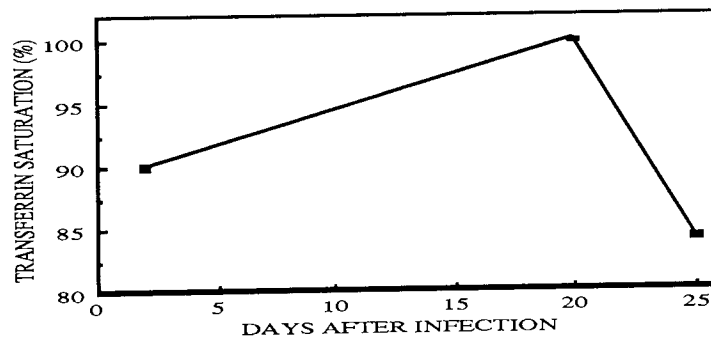
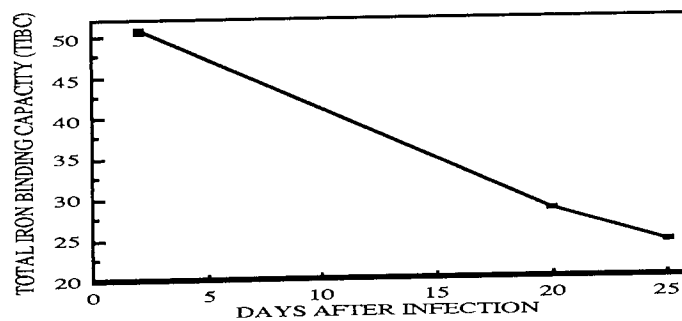


Fig 6.3.7.4



Progression of serum iron concentration (Fig 6.3.7.1), serum transferrin level (Fig 6.3.7.2), transferrin saturation (Fig 6.3.7.3) and TIBC (Fig 6.3.7.4) with infection for patient ECFA.

Fig 6.3.8.1

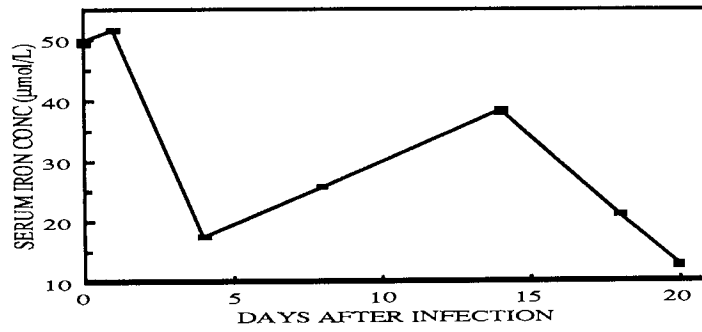


Fig 6.3.8.2

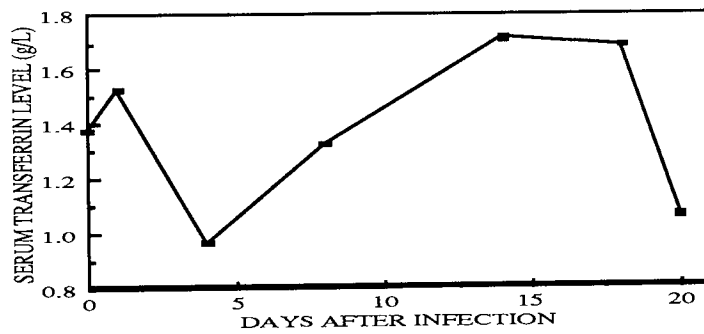


Fig 6.3.8.3

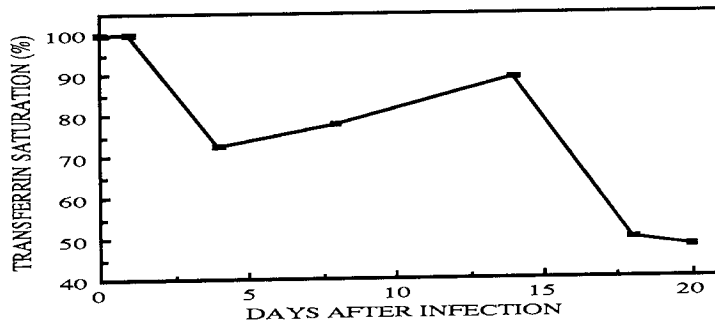
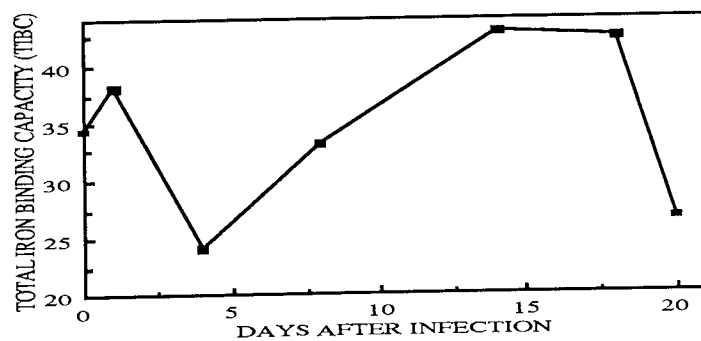


Fig 6.3.8.4



Progression of serum iron concentration (Fig 6.3.8.1), serum transferrin level (Fig 6.3.8.2), transferrin saturation (Fig 6.3.8.3) and TIBC (Fig 6.3.8.4) with infection for patient ECHH1.

Fig 6.3.9.1

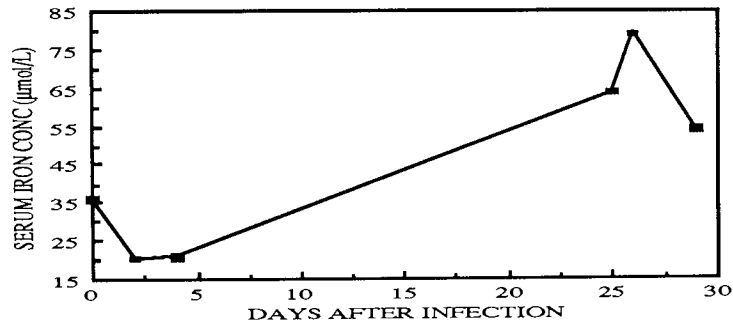


Fig 6.3.9.2

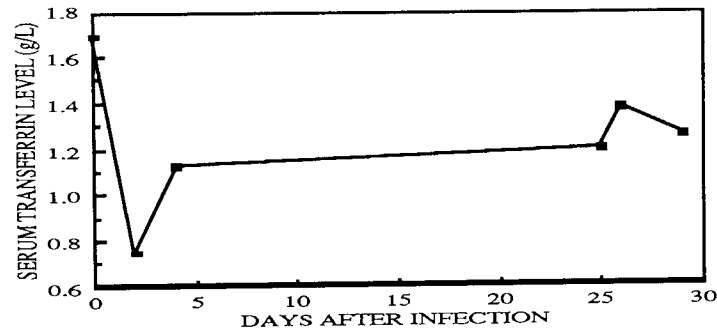


Fig 6.3.9.3

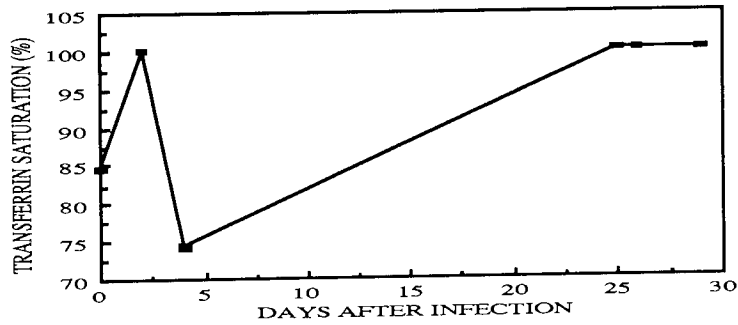
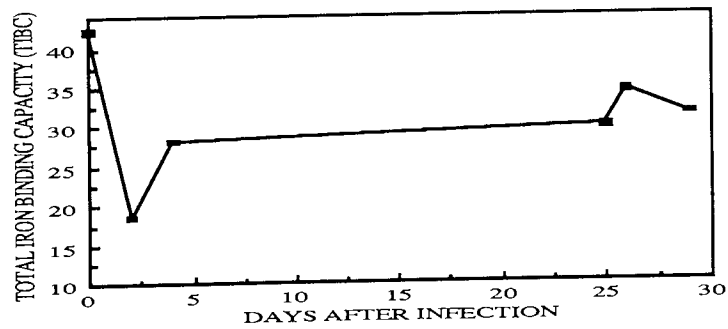


Fig 6.3.9.4



Progression of serum iron concentration (Fig 6.3.9.1), serum transferrin level (Fig 6.3.9.2), transferrin saturation (Fig 6.3.9.3) and TIBC (Fig 6.3.9.4) with infection for patient ECHH2.

Fig 6.3.10.1

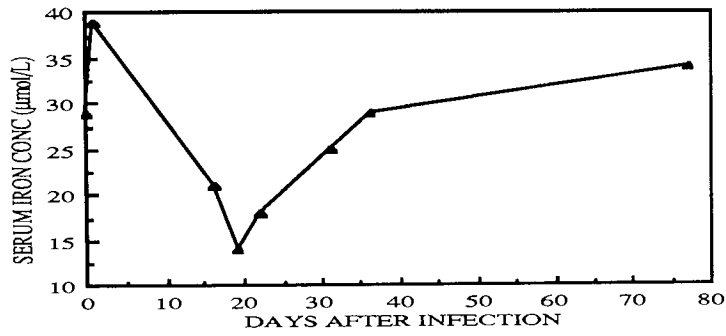


Fig 6.3.10.2

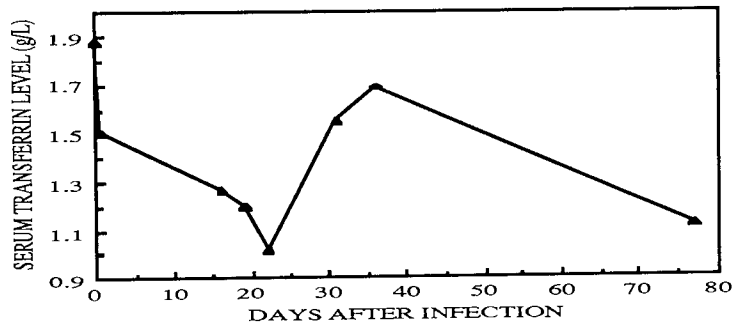


Fig 6.3.10.3

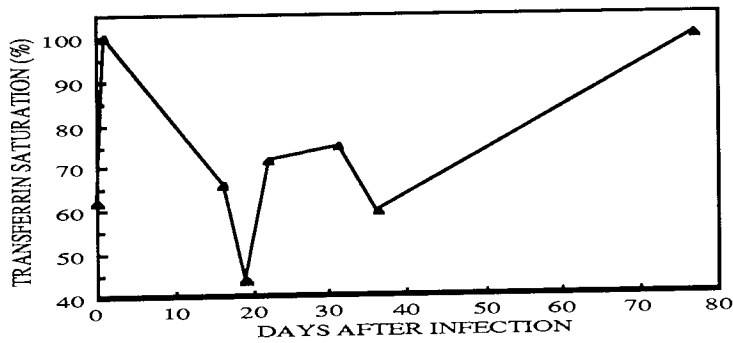
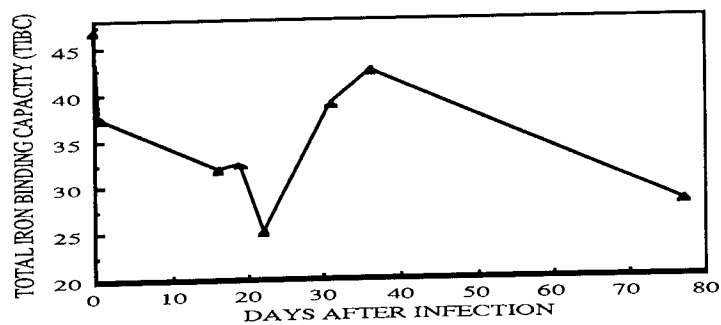


Fig 6.3.10.4



Progression of serum iron concentration (Fig 6.3.10.1), serum transferrin level (Fig 6.3.10.2), transferrin saturation (Fig 6.3.10.3) and TIBC (Fig 6.3.10.4) with infection for patient KPSW.

Fig 6.3.11.1

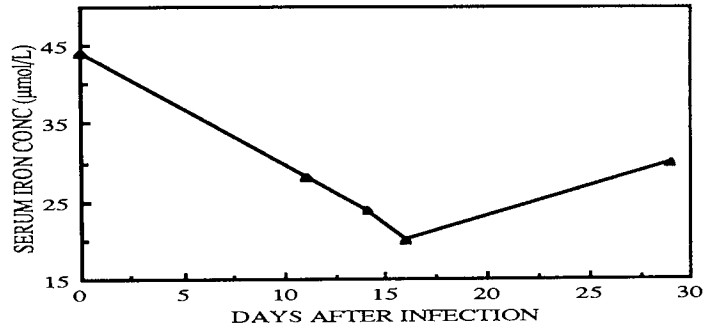


Fig 6.3.11.2

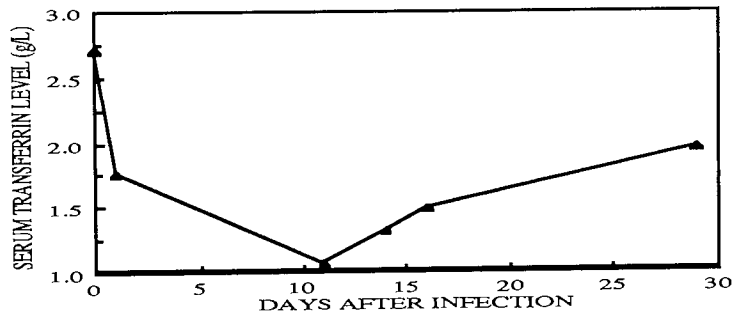


Fig 6.3.11.3

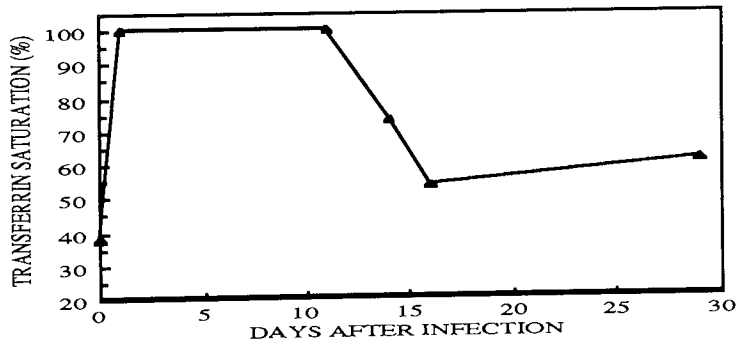
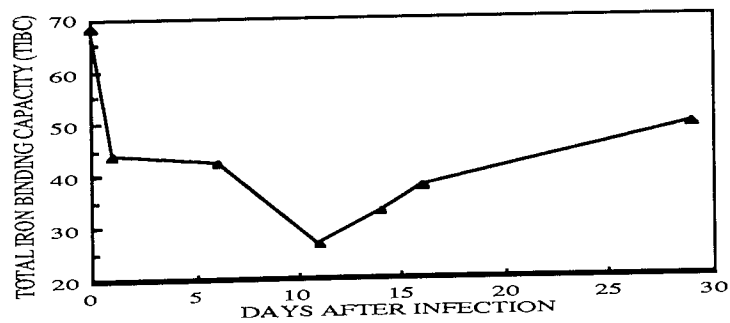


Fig 6.3.11.4



Progression of serum iron concentration (Fig 6.3.11.1), serum transferrin level (Fig 6.3.11.2), transferrin saturation (Fig 6.3.11.3) and TIBC (Fig 6.3.11.4) with infection for patient KPWS.

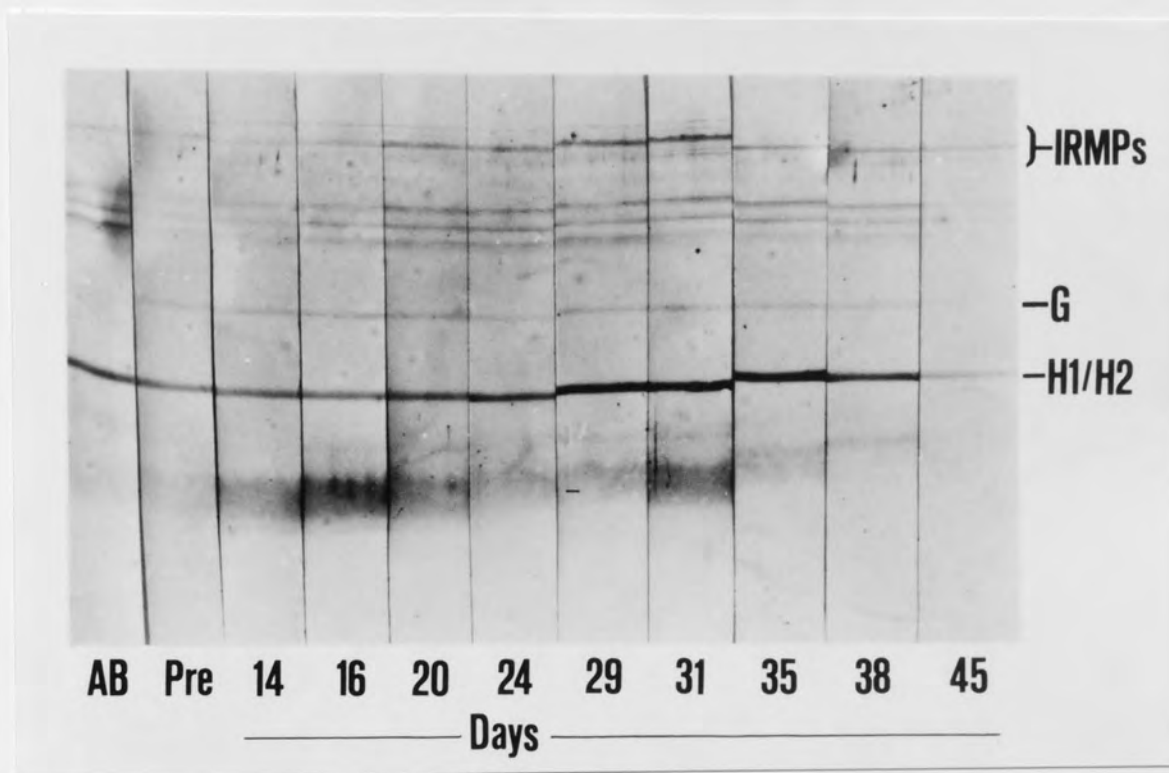
6.4 REACTION OF ANTIBODIES IN THE SERA FROM LEUKAEMIC PATIENTS WITH OM ANTIGENS OF *P. aeruginosa*, *E. coli* AND *K. pneumoniae*.

Immunoblotting with sera from leukaemic patients were used to investigate the antibody response to OM antigens of *P. aeruginosa*, *E. coli* and *K. pneumoniae*. The recognition of OM antigens by serum IgG antibodies are shown pre-infection, throughout the infection episode and where possible on recovery.

6.4.1 Antibody response to *P. aeruginosa*.

Figure 6.4.1.1 shows those antigens of *P. aeruginosa* recognised by IgG antibodies in the serum of patient PAVI. One of the first OMP to be recognised was protein H, although it is not clear whether the strong response observed is to H1 (OprH) or H2 (OprL), because the individual proteins were not separated in this system. The results indicate that the patient had antibodies to IRMPs, proteins D, E (OprE), F (OprF) and G (OprG). For PAJK (Fig. 6.4.1.2) a marked response was observed to IRMPs (75-90 kDa), indeed these represented the most intense bands on the immunoblot. A band with a molecular weight of approximately 55 kDa postulated to be the flagella protein was also strongly recognised (marked with arrow). The band in question was not as distinct in the coomassie blue-stained gel (data not shown), presumably because the amount of flagellin present was below the sensitivity of the protein stain. Serum IgG antibodies additionally detected protein D but, the response to OprE, OprH and OprL was very weak and no detectable reaction could be observed for OprF. The serum antibody response for patient PAGF (Fig. 6.4.1.3) shows a strong reaction with the flagella protein (marked with arrow). OprH was also detected, with OprL being recognised by day 4. Interestingly, the IRMPs were only faintly detected in this patient, who unfortunately died on day 4. This lack of recognition of IRMPs has been noted previously, in mitomycin C treated and untreated cells of *P. aeruginosa* 6750 probed with sera from a leukaemic patient 14 days after initial signs of infection (Fig 5.4, chapter 5).

Fig 6.4.1.1



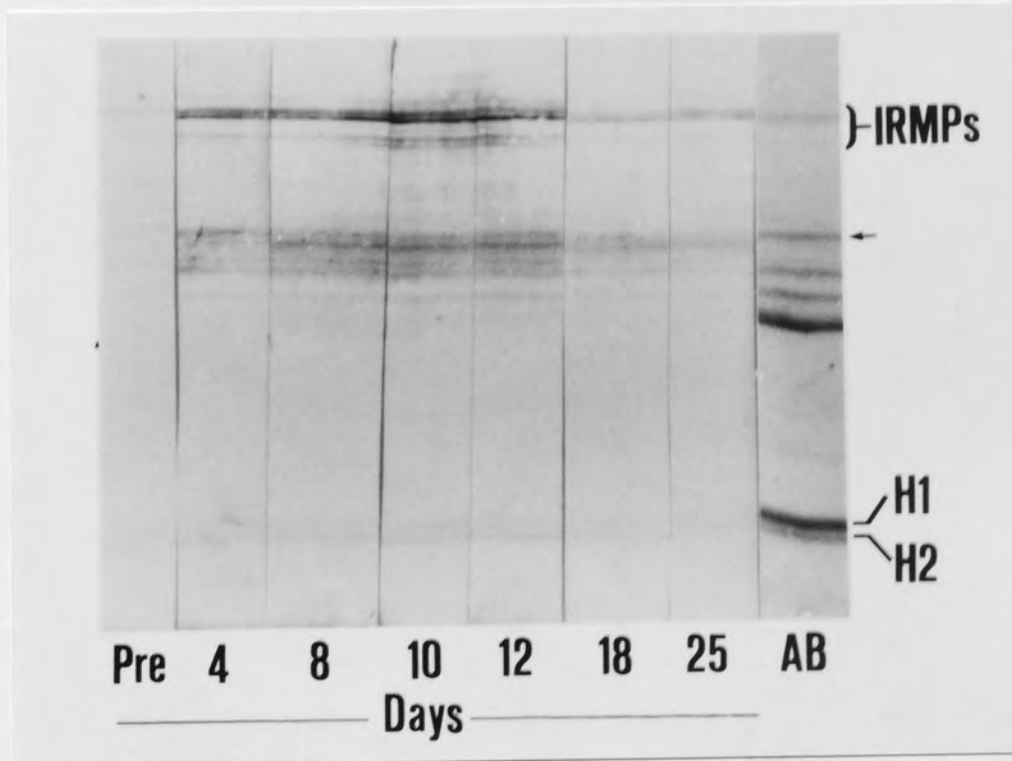
Strip immunoblots of *P. aeruginosa* PAVI OMPs grown in CDM-Fe, electrophoretically transferred to NC paper and probed with sera taken sequentially from a leukaemic patient throughout an infection episode.

'AB' refers to a replicate NC strip stained with amido black 1%w/v.

'Pre' refers to pre-infection serum.

'Days' refer to number of days after onset of infection.

Fig 6.4.1.2



Strip immunoblots of *P. aeruginosa* PAJK OMPs grown in CDM-Fe, electrophoretically transferred to NC paper and probed with sera taken sequentially from a leukaemic patient throughout an infection episode.

'Pre' refers to pre-infection serum.

'AB' refers to a replicate NC strip stained with amido black 1%w/v.

'Days' refer to number of days after onset of infection.

The arrow refers to the flagella protein.

Fig 6.4.1.3



Strip immunoblots of *P. aeruginosa* PAGF OMPs grown in CDM-Fe, electrophoretically transferred to NC paper and probed with sera taken sequentially from a leukaemic patient throughout an infection episode.

'AB' refers to a replicate NC strip stained with amido black 1%w/v.

'Days' refer to number of days after onset of infection.

The arrow refers to the flagella protein.

6.4.2 Antibody response to *E. coli*.

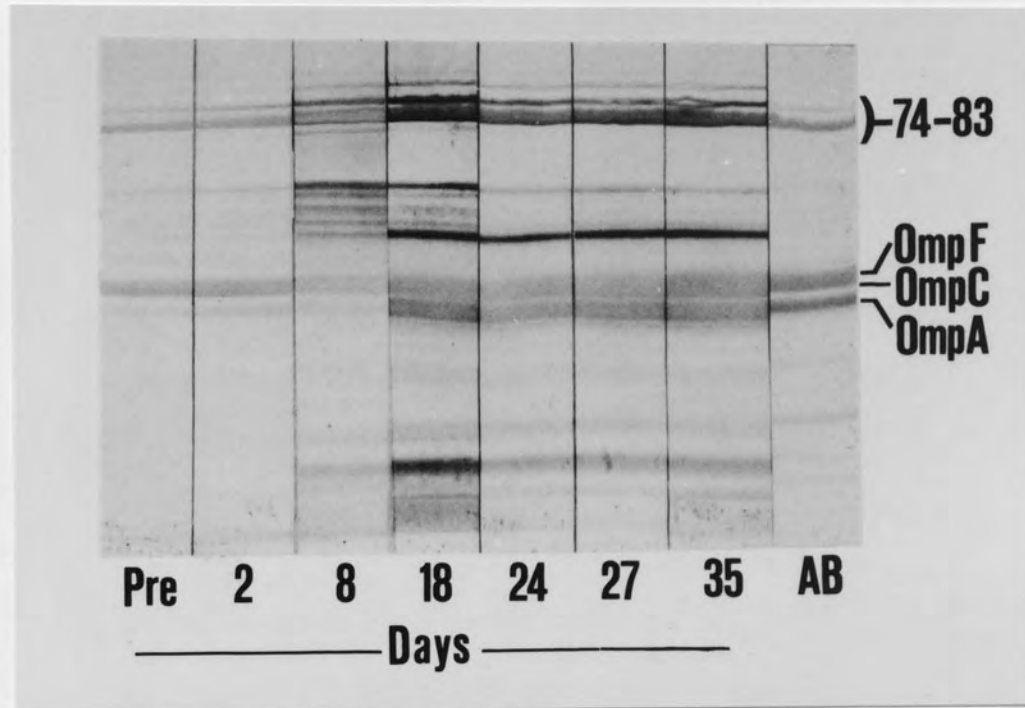
The antigens of *E. coli* recognised by IgG antibodies in the sera of leukaemic patients were examined. Strip immunoblotting was performed to measure any changes in antibody response prior, during and in post-infection convalescence. Figure 6.4.2.1 shows the patient ECSM, the peak immune response occurring on day 18. Serum IgG antibodies showed a strong reaction with the IRMPs (74-83 kDa), the 17.5 kDa protein and additionally with OMPs with molecular weights of approximately 47 kDa (situated above OmpF). The OmpA protein was only recognised 18 days after diagnosis of infection, whereas OmpF and OmpC were recognised throughout. Similar results were found for ECVI (Fig. 6.4.2.2) and ECHD (Fig. 6.4.2.3). A particularly strong IgG antibody response can be observed for the former patient (ECVI), who previously suffered a *P. aeruginosa* septicaemia (Fig. 6.4.1.1). Figures 6.4.2.4, 6.4.2.5 and 6.4.2.6 show the strip immunoblots for the patients ECFA, ECHH1 (first infection) and ECHH2 (second infection), respectively. No response to IRMPs were detected by IgG antibodies in convalescent sera from these leukaemic patients. The major immune response being directed against the porin proteins as observed for previous patients. Despite an initial response by ECHH1 (Fig. 6.4.2.5), days 4 to 8 represented a weak response to the OMP antigens, before a stronger reaction with the porin protein was observed.

6.4.3 Antibody response to *K. pneumoniae*.

Two leukaemic patients with septicaemia, namely KPSW (Fig 6.4.3.1) and KPWS (Fig 6.4.3.2) showed a very strong IgG response to the surface antigens of *K. pneumoniae*. The response found with the pre-infection serum sample (particularly in Fig. 6.4.3.2) taken at diagnosis of septicaemia can be interpreted in two ways. Firstly, the patient may have been exposed to *K. pneumoniae* prior to the diagnosis of septicaemia, by, for example, a sub-clinical local infection, and therefore have already started to generate an immune response, or, secondly, the organism may have been part of the normal flora.

Both patients sera contained IgG antibodies that reacted very strongly with the IRMPs (69-83 kDa) although this did not occur until day 22 with KPSW (Fig 6.4.3.1). The

Fig 6.4.2.1



Strip immunoblots of *E. coli* ECSM OMPs grown in CDM-Fe, electrophoretically transferred to NC paper and probed with sera taken sequentially from a leukaemic patient throughout an infection episode.

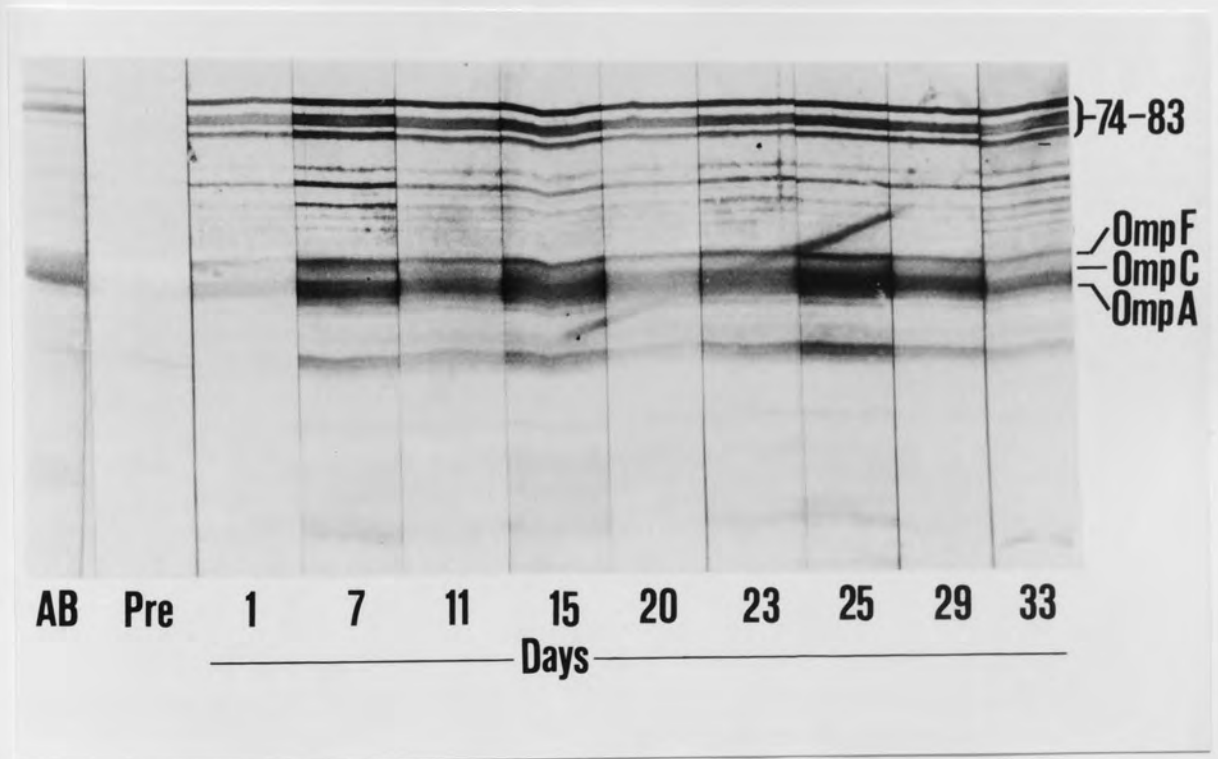
'Pre' refers to pre-infection serum.

'AB' refers to a replicate NC strip stained with amido black 1%w/v.

'Days' refer to number of days after onset of infection.

Molecular weights are in Kilodaltons.

Fig 6.4.2.2



Strip immunoblots of *E. coli* ECVI OMPs grown in CDM-Fe, electrophoretically transferred to NC paper and probed with sera taken sequentially from a leukaemic patient throughout an infection episode.

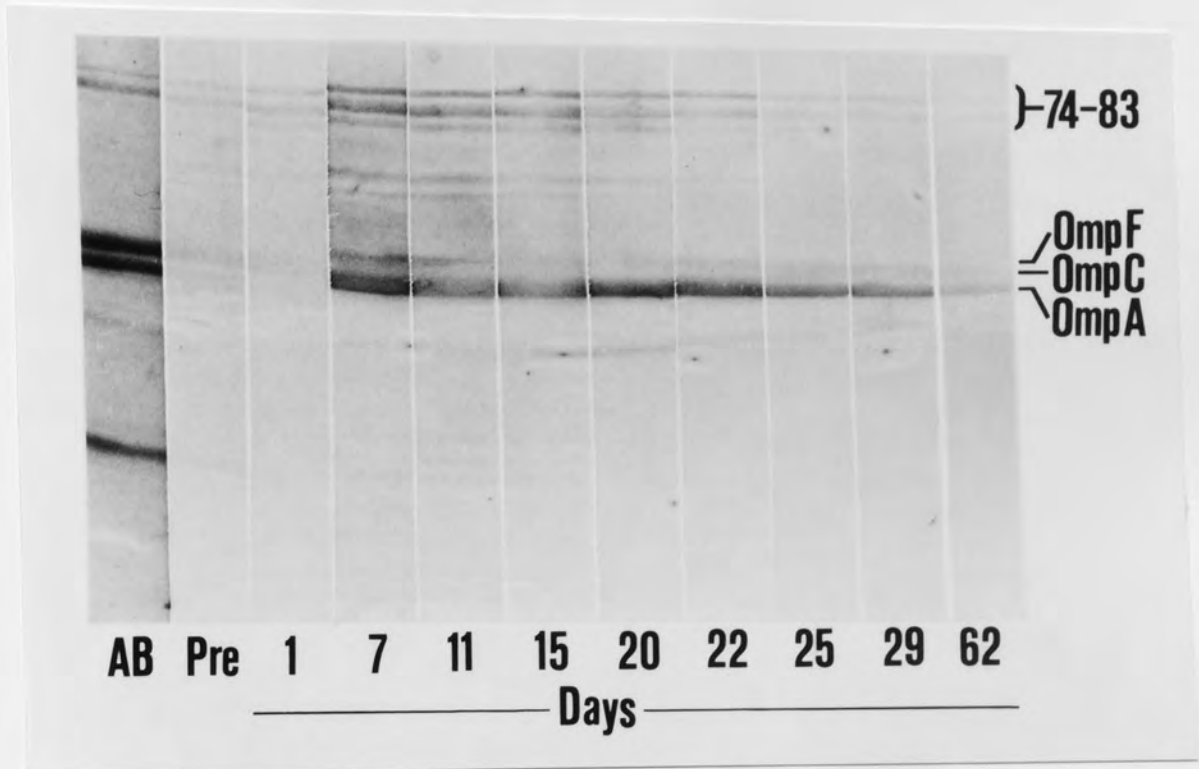
'AB' refers to a replicate NC strip stained with amido black 1%w/v.

'Pre' refers to pre-infection serum.

'Days' refer to number of days after onset of infection.

Molecular weights are in Kilodaltons.

Fig 6.4.2.3



Strip immunoblots of *E. coli* ECHD OMPs grown in CDM-Fe, electrophoretically transferred to NC paper and probed with sera taken sequentially from a leukaemic patient throughout an infection episode.

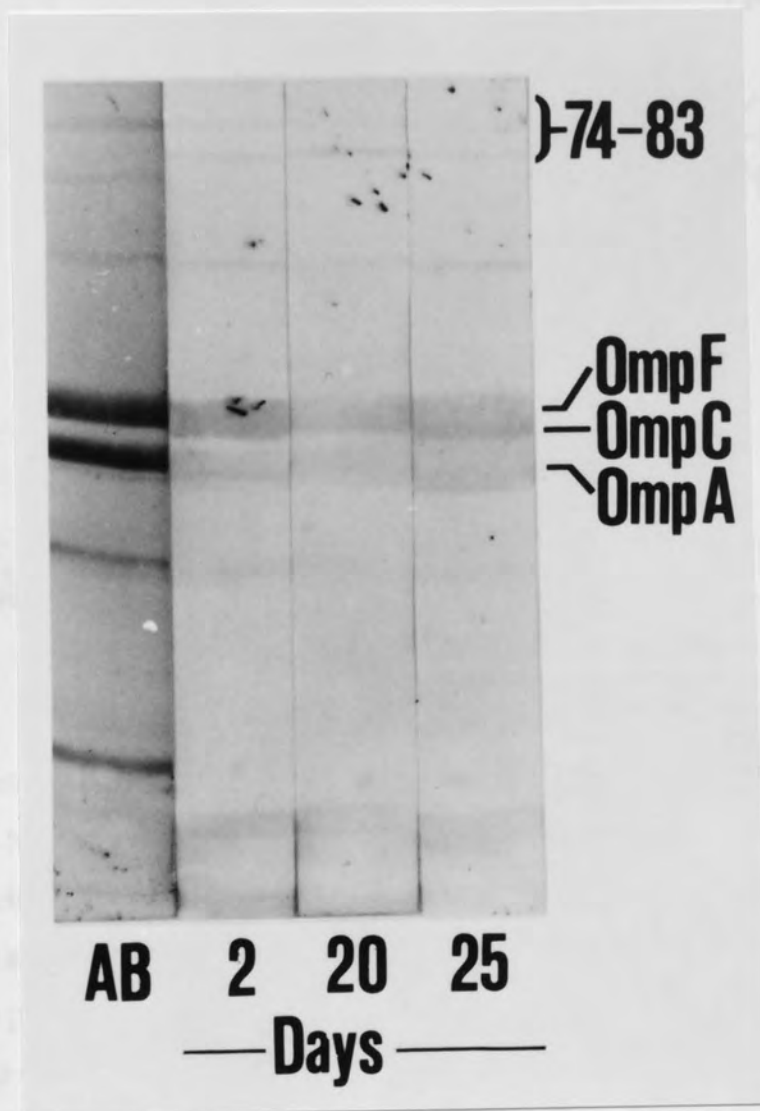
'AB' refers to a replicate NC strip stained with amido black 1%w/v.

'Pre' refers to pre-infection serum.

'Days' refer to number of days after onset of infection.

Molecular weights are in Kilodaltons.

Fig 6.4.2.4



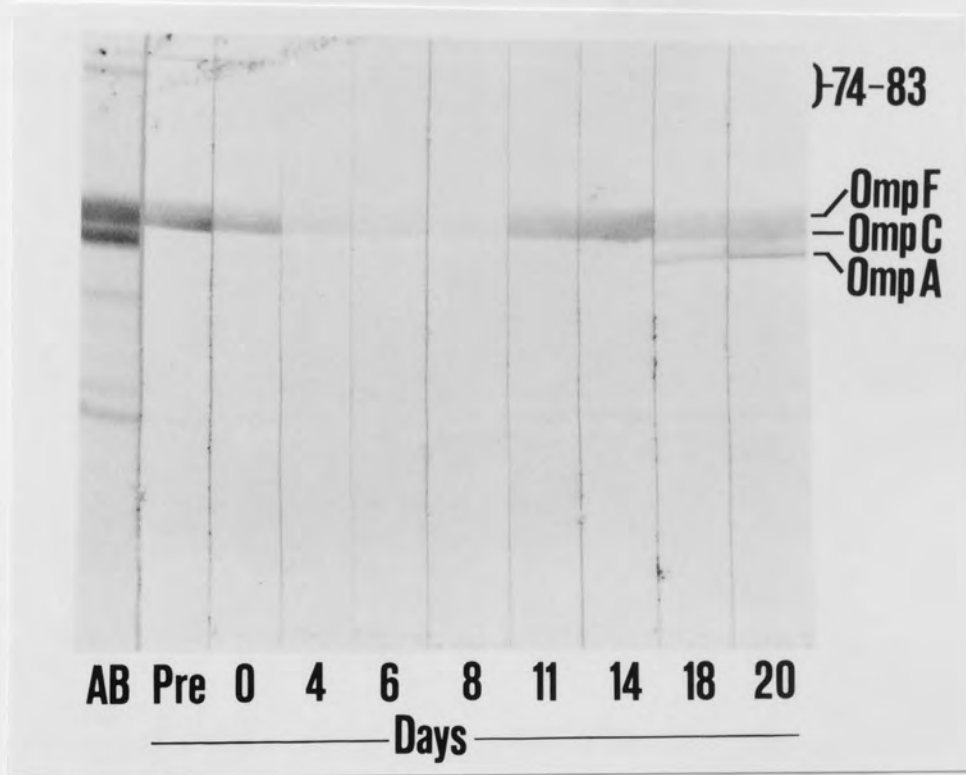
Strip immunoblots of *E. coli* ECFA OMPs grown in CDM-Fe, electrophoretically transferred to NC paper and probed with sera taken sequentially from a leukaemic patient throughout an infection episode.

'AB' refers to a replicate NC strip stained with amido black 1%w/v.

'Days' refer to number of days after onset of infection.

Molecular weights are in Kilodaltons.

Fig 6.4.2.5



Strip immunoblots of *E. coli* ECHH1 OMPs grown in CDM-Fe, electrophoretically transferred to NC paper and probed with sera taken sequentially from a leukaemic patient throughout an infection episode.

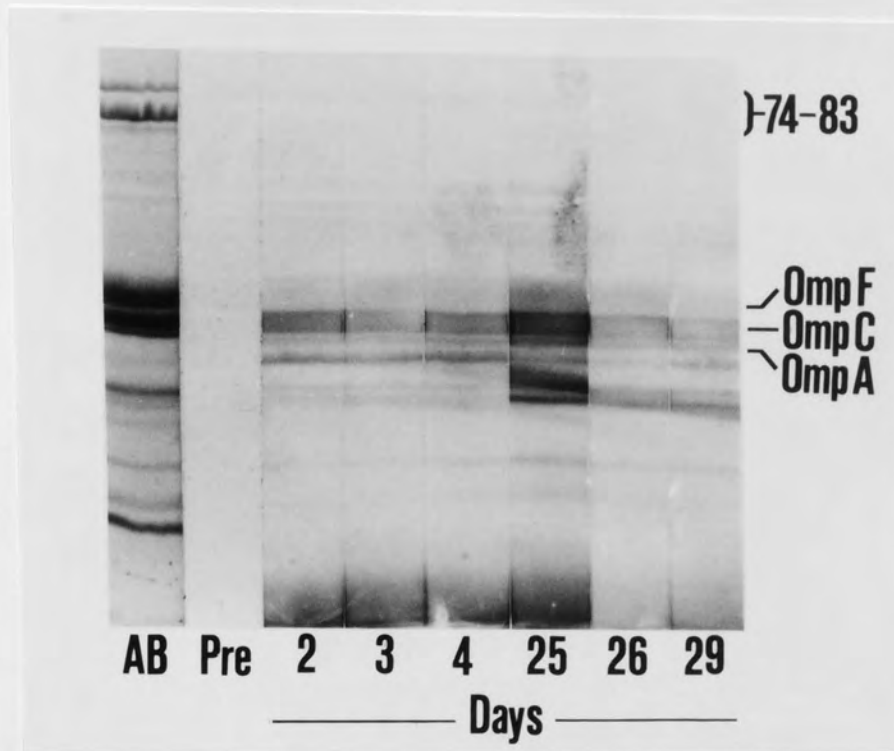
'AB' refers to a replicate NC strip stained with amido black 1%w/v.

'Pre' refers to pre-infection serum.

'Days' refer to number of days after onset of infection.

Molecular weights are in Kilodaltons.

Fig 6.4.2.6



Strip immunoblots of *E. coli* ECHH2 OMPs grown in CDM-Fe, electrophoretically transferred to NC paper and probed with sera taken sequentially from a leukaemic patient throughout an infection episode.

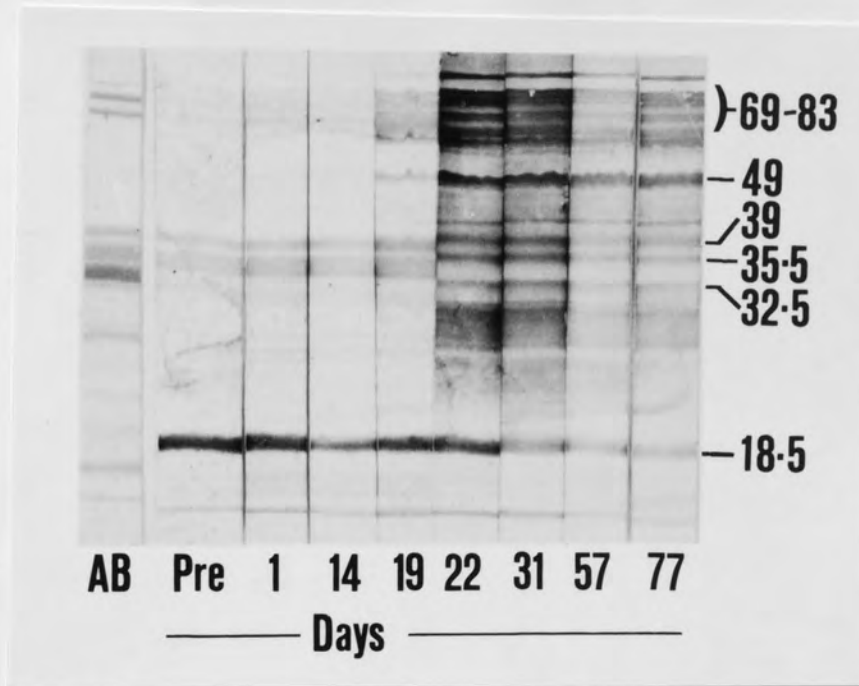
'AB' refers to a replicate NC strip stained with amido black 1%w/v.

'Pre' refers to pre-infection serum.

'Days' refer to number of days after onset of infection.

Molecular weights are in Kilodaltons.

Fig 6.4.3.1



Strip immunoblots of *K. pneumoniae* KPSW OMPs grown in CDM-Fe, electrophoretically transferred to NC paper and probed with sera taken sequentially from a leukaemic patient throughout an infection episode.

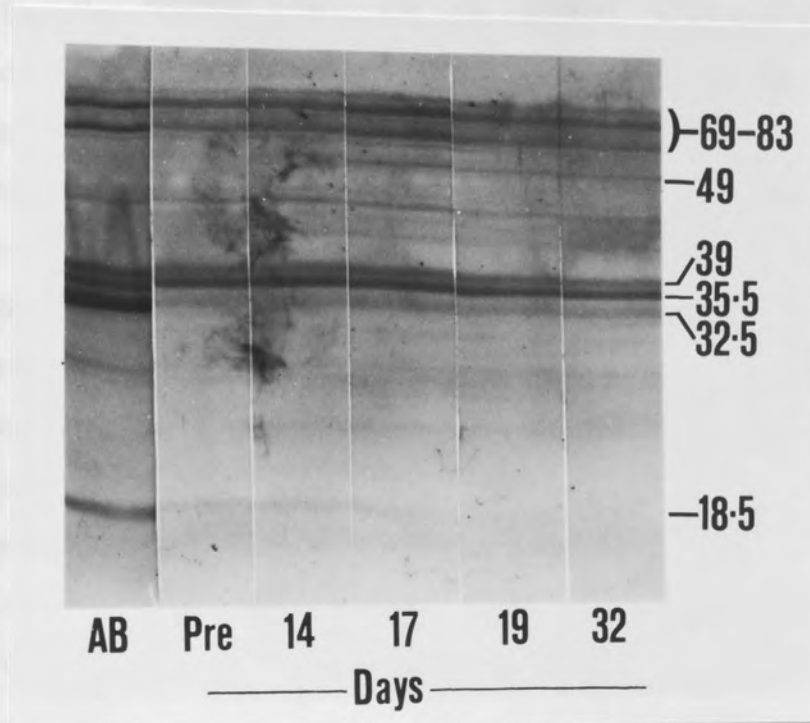
'AB' refers to a replicate NC strip stained with amido black 1%w/v.

'Pre' refers to pre-infection serum.

'Days' refer to number of days after onset of infection.

Molecular weights are in Kilodaltons.

Fig 6.4.3.2



Strip immunoblots of *K. pneumoniae* KPWS OMPs grown in CDM-Fe, electrophoretically transferred to NC paper and probed with sera taken sequentially from a leukaemic patient throughout an infection episode.

'AB' refers to a replicate NC strip stained with amido black 1%w/v.

'Pre' refers to pre-infection serum.

'Days' refer to number of days after onset of infection.

Molecular weights are in Kilodaltons.

major protein bands 35.5 kDa and 39 kDa were found to be highly immunogenic throughout the infection episode. These proteins have been found to be peptidoglycan associated (Lodge, 1987) and it is proposed that these proteins are functionally related to the OmpF and OmpC porin proteins of *E. coli* with which they cross-react immunologically (Hofstra and Dankert, 1979). The third major OMP 32.5 kDa was detected only faintly. There was a strong reaction with the 49 kDa protein particularly for KPSW (Fig 6.4.3.1) occurring by day 19 after diagnosis. This protein has been observed previously when *K. pneumoniae* was grown in carbon limited chemostat cultures (Lodge, 1987). A similar protein at 48 kDa has been described which was present in comparable amounts whether the carbon source was glucose, glycerol or maltose (Sterkenburg *et al.*, 1984). These authors demonstrated that in general, limitation specific proteins were not affected by the form in which the growth limiting nutrient was provided, it may be organic, negatively or positively charged inorganic. This together with the fact that the 49 kDa protein is not peptidoglycan associated, indicates that this protein does not function as a specific pore but is involved in optimising the uptake of the limiting nutrient (Lodge, 1987).

6.5 ANTIBODY RESPONSE TO WHOLE CELL SURFACE ANTIGENS EXAMINED BY ELISA.

The whole bacterial cell ELISA measures antibody binding to surface-exposed antigenic determinants, both protein and LPS (Borowski *et al.*, 1984). Initially IgG, IgM and IgA titres in sera from leukaemic patients infected with *K. pneumoniae* grown under CDM+Fe and CDM-Fe were quantified (Fig 6.5.3.1-3). All the titres are expressed as the reciprocal of the serum dilution which gave an absorbance of 0.1 units above blank wells (see section 3.5.2). Comparison of end-point titres indicated that IgG represented the major class of immunoglobulins present in the sera of leukaemic patients. Figure 6.5.3.2 shows a large increase in the IgM antibody titre, however this response is transient, being superseded by the IgG antibody titre (Fig 6.5.3.1). Titres of IgA in serum to KPSW (Fig 6.5.3.3) surface antigenic determinants were more than 10-fold lower than either IgG or IgM. The end-point titres to CDM-Fe grown antigens were higher than CDM+Fe grown cells (data not shown). Therefore, ELISA experiments were conducted under iron depleted conditions using IgG immunoglobulin.

6.5.1 Antibody titre to *P. aeruginosa*.

Figures 6.5.1.1, 6.5.1.2 and 6.5.1.3 show the titration curves of IgG in sequential sera from leukaemic patients to whole cell surface antigens of *P. aeruginosa* grown in CDM-Fe for the patients PAVI, PAJK and PAGF, respectively. Despite the initial reduction for PAVI (Fig 6.5.1.1), the IgG titres increased throughout the infection episode, although this increase is comparatively small particularly for the patient PAGF (Fig 6.5.1.3). These ELISA data reflect the results obtained by immunoblotting.

6.5.2 Antibody titre to *E. coli*.

Antibody production to whole cells of *E. coli* for patients ECSM, ECVI, ECHD, ECFA, ECHH1 and ECHH2 are shown (Figs 6.5.2.1 to 6.5.2.6, respectively). The serum IgG titres were found to increase during the infection period and decrease on recovery. The weak immune response observed from day 4-8 for the patient ECHH1 (Fig 6.4.2.5) is replicated by the ELISA results (Fig 6.5.2.5). In addition, despite the generally low antibody activity obtained for leukaemic patients, immunoblotting analysis correlated to the ELISA results.

6.5.3 Antibody titre to *K. pneumoniae*.

Figures 6.5.3.1 and 6.5.3.4 show the IgG antibody response to whole cells of *K. pneumoniae* for the patients KPSW and KPWS, respectively. Similar results to *P. aeruginosa* and *E. coli* were obtained, the IgG titre increased as the infection proceeded, but again, due to treatment with antineoplastic agents these patients in general exhibited a poor antibody response to infection. A good correlation was observed with the immunoblotting analysis. Figures 6.5.3.2 and 6.5.3.3 show the IgM and IgA antibody response, respectively for the patient KPSW, showing IgG immunoglobulin to represent the main immunogenic challenge in the sera of leukaemic patients.

Fig 6.5.1.1

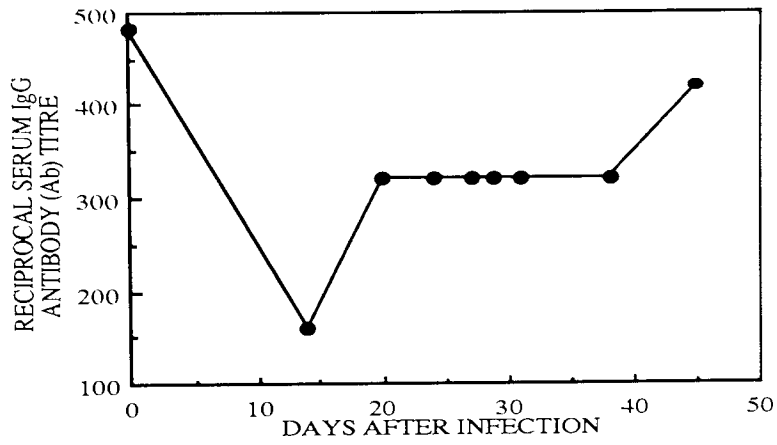


Fig 6.5.1.2

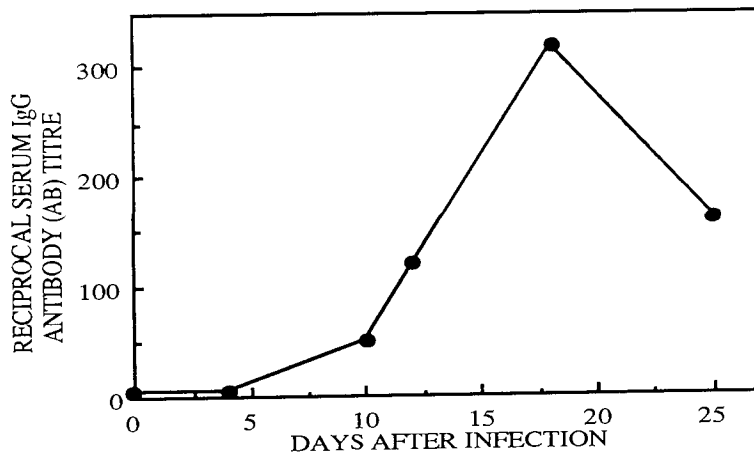
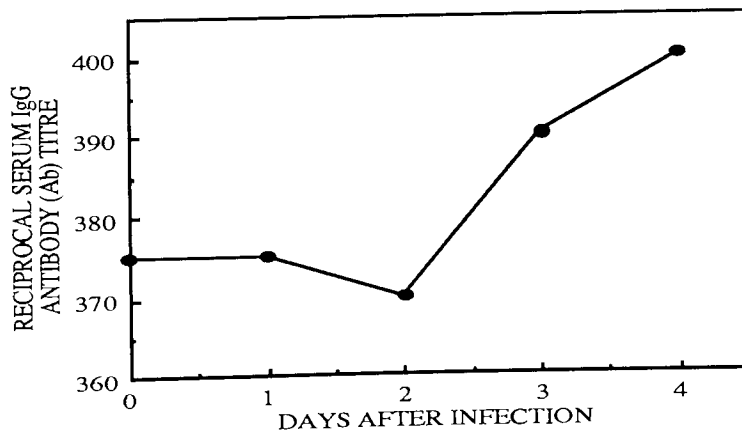


Fig 6.5.1.3



Progression of antibody titre (IgG) with infection for patients PAVI (Fig 6.5.1.1), PAJK (Fig 6.5.1.2) and PAGF (Fig 6.5.1.3).

Fig 6.5.2.1

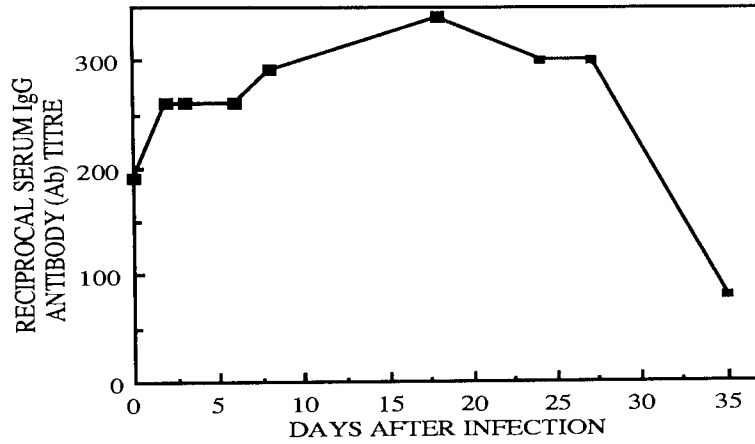


Fig 6.5.2.2

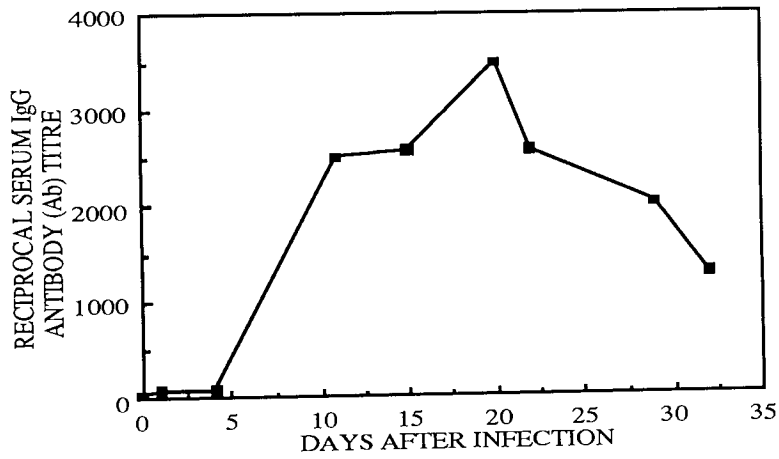
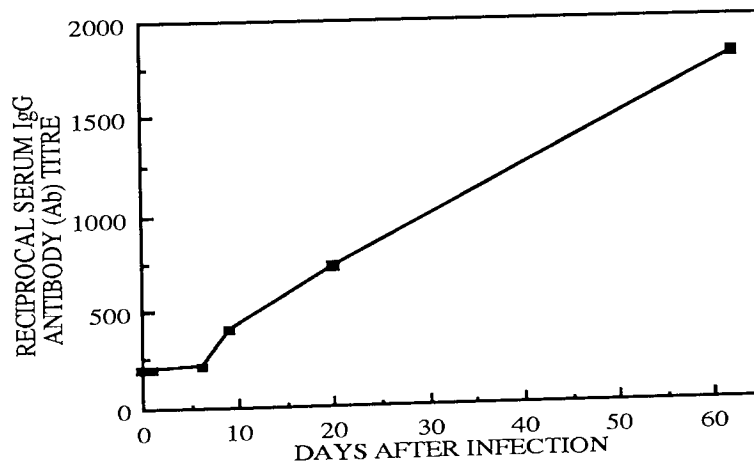


Fig 6.5.2.3



Progression of antibody titre (IgG) with infection for patients ECSM (Fig 6.5.2.1), ECVI (Fig 6.5.2.2) and ECHD (Fig 6.5.2.3).

Fig 6.5.2.4

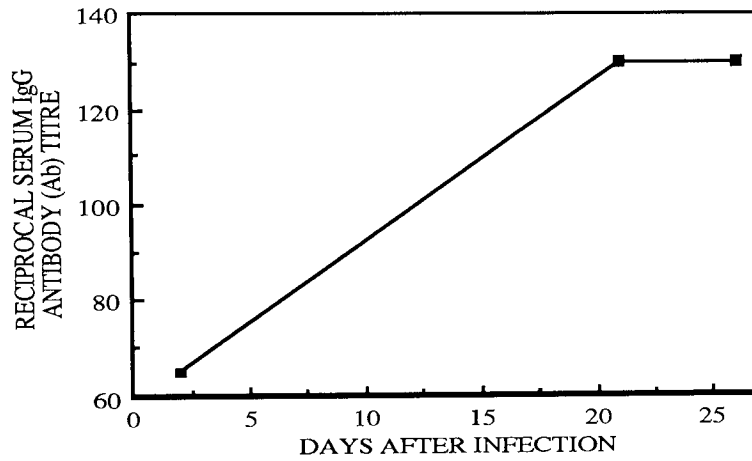


Fig 6.5.2.5

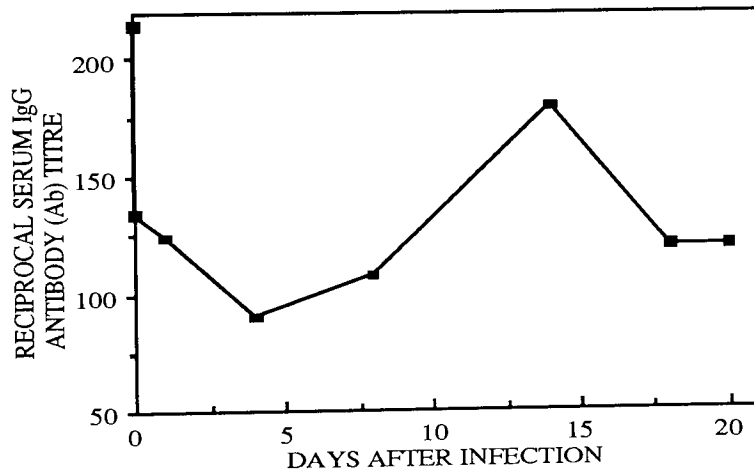
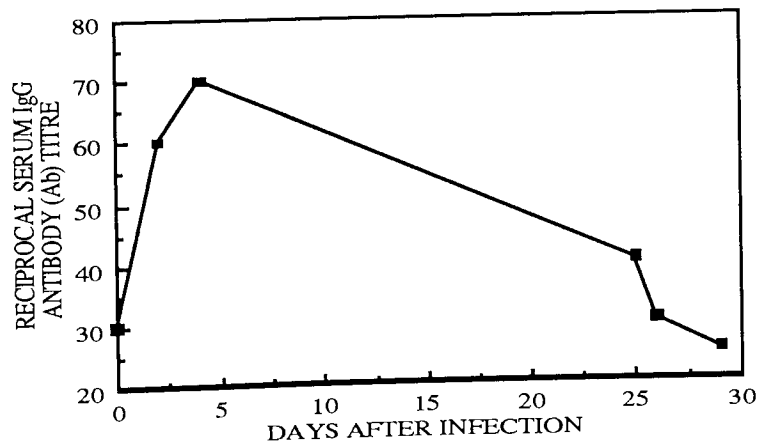


Fig 6.5.2.6



Progression of antibody titre (IgG) with infection for patients ECFA (Fig 6.5.2.4), ECHH1 (Fig 6.5.2.5) and ECHH2 (Fig 6.5.2.6).

Fig 6.5.3.1

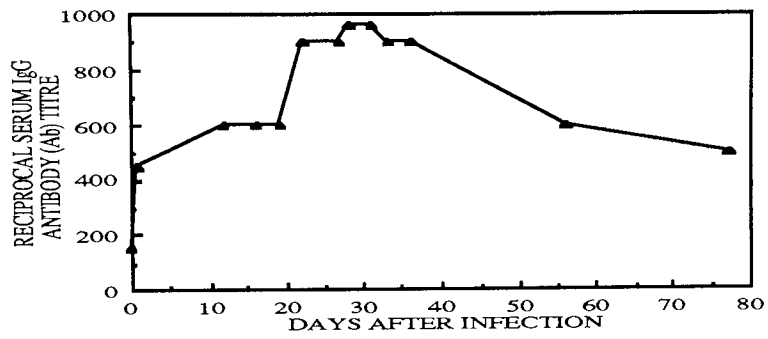


Fig 6.5.3.2

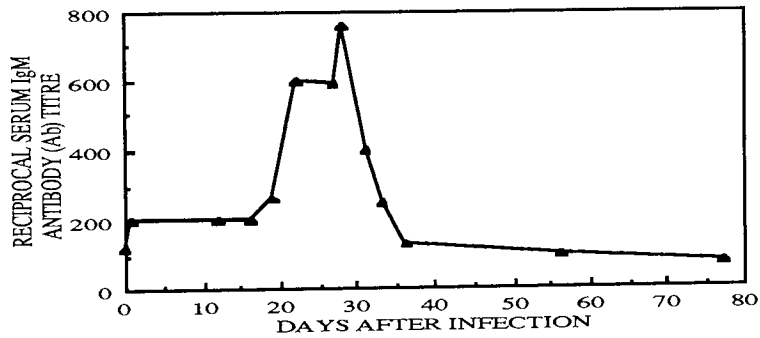


Fig 6.5.3.3

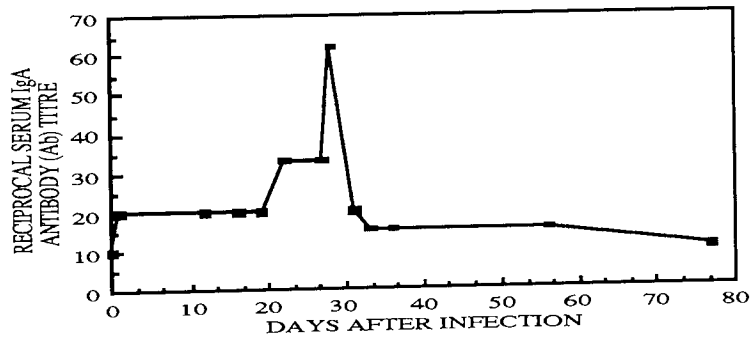
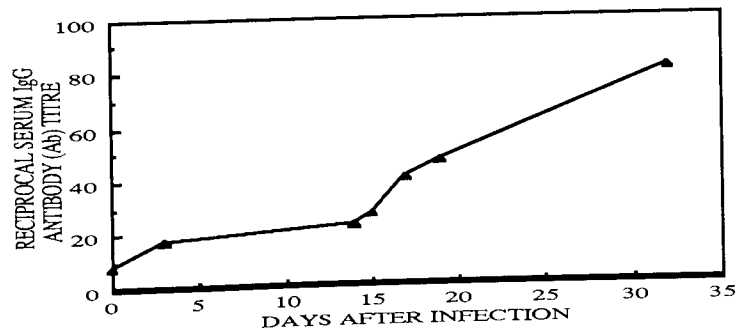


Fig 6.5.3.4



Figs 6.5.3.1, 6.5.3.2 and 6.5.3.3 represent the progression of antibody titre (IgG, IgM and IgA, respectively) with infection for patient KPSW. Fig 6.5.3.4 shows the antibody titre (IgG) for patient KPWS.

6.6 DISCUSSION.

Major obstacles to further progress in the successful management of malignant disease include complications arising from the underlying disease or its therapy. Although considerable progress has been made in its prevention and treatment, infection remains the most frequent serious complication in the leukaemic patient. The present investigation addressed the possible association between iron-related parameters and immune response, prior to, during and on recovery from infection episodes in patients with leukaemia.

A vigorous competition exists between vertebrate hosts and their bacterial and neoplastic invaders for host iron, essential for growth of the invading cells (Sussman, 1974; Weinberg, 1981; 1984; 1986; Finkelstein *et al.*, 1983; Oppenheimer, 1989). The host possess an array of mechanisms that withhold iron (collectively termed “nutritional immunity”; Weinberg, 1975). The ability of the invaders to overcome these mechanisms is an important component of virulence (Weinberg, 1974; 1978). One of the components of “nutritional immunity” is the presence of powerful iron-binding glycoproteins of the transferrin class, at tissue sites that are frequently or constantly threatened with microbial invasion. Additional components which may develop in the infected host include: a) suppression of intestinal absorption of iron, b) decrease in the quantity of iron in the plasma and expansion of the metal in the storage compartment, c) increased mobilisation and/or synthesis of iron-binding proteins and d) suppression of microbial siderophore synthesis by elevation of host body temperature.

Iron-withholding defence is compromised in a diversity of conditions many of which involve aspects of iron over-load in specific tissues. Many investigators have reported the ease with which “nutritional immunity” is overcome, either in extracted body fluids or hosts, with small amounts of iron. For example, the antimicrobial power of serum *in vitro* is overcome merely by adding sufficient iron to attain a saturation of transferrin of about 60% (Weinberg, 1975). In *in vivo* studies, the number of cells of *P. aeruginosa* needed to kill 50% of test rats or mice, compared with controls was lowered significantly, simply by concurrent intravenous injection of 1 to 5mg of iron per kilogram of body weight (Weinberg, 1975).

The role of iron and iron-binding proteins in host resistance to clinical infection has been controversial. Different authors claim either that iron deficiency helps, the “nutritional immunity” hypothesis (Weinberg, 1974; 1986; Murray *et al.*, 1978), or hinders defences against infection (Chandra, 1990). This is not surprising since host defences are complex and it is seldom possible to isolate the effects of a single component in clinical disease. Numerous experimental studies have shown that manipulation of iron availability can affect the growth of microorganisms *in vitro* and *in vivo* and provides evidence that iron administration might actually be harmful by predisposing to infection (Murray *et al.*, 1978; Weinberg, 1978; Bullen, 1981; Dallman, 1987; Bullen *et al.*, 1991).

Clinical investigators, on the other hand, have argued that these factors play, at most, a minor role in host resistance to clinical infections (Weinberg, 1977; Hershko *et al.*, 1988; Chandra, 1990). They observed that there is no simple relationship between serum or total body iron levels and susceptibility to infections. Patients with hyperferraemia due to thalassemia or haemochromatosis do not have excessive infections (although this is disputed by many authors; Chart and Griffiths, 1985; Brock, 1986; Kluger and Bullen, 1987; Bullen *et al.*, 1991). Furthermore, most of the patients with infections and hyperferraemia have conditions that also produce neutropenia and immunosuppression. Indeed, it could be argued that neutropenia does play a role in reducing resistance, particularly in the leukaemic host, where profound neutropenia often results from chemotherapy treatment and therefore is a contributing factor. Many investigators have concluded that these are important components in host resistance and changes in iron levels are minor contributors (Hershko *et al.*, 1988). However, it has been shown that even with normal polymorphs saturation of the serum transferrin can lead to rapid extracellular growth, which eventually overwhelms the phagocytic cells (Bullen *et al.*, 1974; 1991; Ward *et al.*, 1986). These authors emphasise the importance of transferrin in restricting the availability of iron which is essential for resistance to infection.

Patients with Hodgkin’s disease, lymphoma and most other malignancies characteristically have reduced serum iron levels and a reduced percentage saturation of transferrin (Roeser, 1980). Hypoferraemia is one of the most common findings of chronic disease (Roeser, 1980). However, present data confirms the work of Caroline *et al.*, (1969) that acute

leukaemia appears to be an exception, hyperferraemic episodes with iron saturation values more than 50% often occurring in leukaemic patients (Weinberg, 1975; Kluger and Bullen, 1987). Persons who have episodes of hyperferraemia or hypotransferrinaemia are more susceptible to bacterial pathogens at these times than during periods when their iron saturation of transferrin is within normal limits (Weinberg, 1974; 1975).

Patients with leukaemia are unusually susceptible to infection (Caroline *et al.*, 1969; Estey *et al.*, 1982; Hunter *et al.*, 1984a; Griffiths and Bullen, 1987). Estey *et al.*, (1982) recorded that of 161 failures in the treatment of 378 patients with leukaemia, 36 were considered therapeutic failures and in the rest (125) death was largely attributable to infection. The bacteria involved included *Klebsiella* and *Pseudomonas* species. Hunter *et al.*, (1984b) found that 28 out of 47 adults (60%) with leukaemia had levels of transferrin saturation above 50%. It was shown that the highly saturated sera had far less ability to inhibit a strain of *P. aeruginosa* than did normal sera and that the higher the saturation the less the inhibition. Additionally, some patients exhibited low levels of transferrin, this was also observed in 6 of 20 (30%) patients in phase 1 of this study and in 9 out of 11 (82%) of cases at onset of infection.

In this study, the iron status of 20 newly diagnosed leukaemic patients showed that 55% had percentage saturation of transferrin with iron greater than 50%, only 7 (35%) of the patients had all the values within the normal range. Assessment of iron-related parameters during infection showed that all patients exhibited abnormally high serum iron levels and 100% transferrin saturation during the initial stages of infection. Caroline *et al.*, (1969) showed that of 34 patients with leukaemia, 21 had high levels of serum iron and in 17 patients, the transferrin was fully saturated. The authors (1969) found that leukaemic sera (96-100% saturation) was far less able to inhibit *C. albicans* than normal sera and gave counts approximately 1000 times greater after 48h of incubation. The addition of human transferrin to the leukaemic sera restored its inhibitory power to normal levels. This was reversed by adding iron. These results, together with those obtained in this study suggest that sera with highly saturated transferrin may predispose leukaemic patients to infection.

Hunter *et al.*, (1984b) have suggested that physiological defects resulting in an inability to

modulate iron levels may be more important than those producing hyperferraemia. Moreover, they suggest that protocols designed to demonstrate enhanced infection in animals after parenteral administration of iron reflect acute hyperferraemia rather than chronic hyperferraemia.

Neutropenia and the changes observed in the regulation of serum iron, may both derive from the same abnormalities in bone marrow function (Hunter *et al.*, 1984b). Suppression of bone marrow function sufficient to produce neutropenia, usually produces monocytopenia. Stimulated monocytes produce a mediator called leukocyte endogenous mediator (LEM), which may be the same as endogenous pyrogen or interleukin-1 (Kluger and Bullen, 1987). Among its many effects LEM reduces serum iron at the start of infection (Weinberg, 1975; Kluger and Bullen, 1987). It causes cells of the reticuloendothelial system to withhold iron from transferrin, which brings about its desaturation within hours. In addition, neutrophils produce lactoferrin and it is proposed that it removes iron from transferrin and that the Fe³⁺-loaded lactoferrin is taken up by the macrophages and removed rapidly from the circulation by the reticuloendothelial system (Kluger and Bullen, 1987). It has been shown that lactoferrin does accumulate at sites of inflammation and that, unlike transferrin, whose iron-binding ability is diminished below pH6, it retains its iron-binding properties in the more acidic conditions which often prevail at such locations (Van Snick *et al.*, 1974). Microorganisms are therefore, restricted in iron at sites of infection. Finally, marrow suppression tends to produce hyperferraemia by reducing iron utilisation by erythroid cells (Hunter *et al.*, 1984b). Therefore, suppression of bone marrow function affects host defence mechanisms involving iron deprivation in addition to reducing numbers of phagocytic cells. Hunter *et al.*, (1984b) proposed that patients at highest risk of developing infection are those who have both neutropenia and sustained hyperferraemia.

These data demonstrate that abnormalities in the regulation of serum iron and iron-binding proteins involves at least three types of deficits: a low level of transferrin, a high level of serum iron and an inability to reduce iron levels at the start of infection. In addition, these defects have been associated with a reduced ability of serum to inhibit the growth of a test strain of *P. aeruginosa* (Hunter *et al.*, 1984b). Finally, this study is in agreement with other

investigators who noted that increased iron levels, particularly increased percentage saturation of transferrin with iron, predisposes to infection (Caroline *et al.*, 1969; Blumberg *et al.*, 1981; Hunter *et al.*, 1984b; Kluger and Bullen, 1987). Any condition that fully saturates the iron-binding proteins makes iron freely available to bacteria (Kluger and Bullen, 1987). Even in less severe iron overload, where the transferrin saturation is increased, but is less than total, there may still be an association with enhanced incidence of infection (Brock, 1986; Kluger and Bullen, 1987).

There is increasing evidence that both iron overload and iron deficiency are associated with significant abnormalities of immune function. Cell mediated responses in particular, are susceptible to iron deficiency (Good *et al.*, 1988; Farthing, 1989; Chandra, 1990) since the proliferation of lymphocytes requires acquisition of transferrin bound iron (Brock and Mainou-Fowler, 1983; Brock, 1989a). Human studies have suggested that iron deficiency is associated with an altered response to infection as it impedes optimal proliferation of T cells, such as delayed type hypersensitivity and mitogenic responses (Brock, 1986; Dallman, 1987; Kuvibidila, 1987), although the relationship is complex (Good *et al.*, 1988). Thus, while iron deficiency may increase the host's ability to withhold iron from bacteria, this advantage may be offset by impairment of the immune system (Brock, 1986). Similarly, iron overload has been shown to impair phagocytic function and the generation of antigen-specific immune responses in humans and animals (Good *et al.*, 1988; De Sousa, 1989). In addition, iron overload in animals results in a reduction of the numbers of functional helper precursor cells. If a similar event occurs in humans, iron overload would have a compounding effect *in vivo* on the generation of an immune response (Good *et al.*, 1988). Such an effect may in part explain the strong association between clinical iron overload and susceptibility to infection and neoplasia.

Normal iron balance, therefore, seems to achieve a compromise in which iron is not readily accessible to invading microorganisms, yet is present in sufficient quantity to allow the host's immune system to function optimally. Transferrin plays a key part in both mechanisms, but the importance of changes in its iron saturation in affecting microbial growth *in vivo* is unclear.

The iron status of the host can be considered an important determinant in the course of malignancy, by influencing susceptibility to infection (Caroline *et al.*, 1969; Hunter *et al.*, 1984b), by acting as a favourable growth factor for the transformed cells expressing transferrin receptors (Faulk *et al.*, 1980; Habeshaw *et al.*, 1983; Medeiros *et al.*, 1988) and by suppressing key immune recognition functions (Blumberg *et al.*, 1981). The evidence for the value of withholding iron as a defence against neoplasia has been reviewed by Weinberg (1984). Potaznik *et al.*, (1987) explored the possible association between iron-related parameters and survival in paediatric patients with leukaemia. A significant difference was found between the survival of patients according to whether their first measured transferrin saturation at the time of their diagnosis was greater or less than 36%, with fewer deaths in the group with transferrin saturation less than 36%. Compared with phase 1 of this study, where the iron status of 20 randomly-selected, newly-diagnosed leukaemic patients were assessed, 18 of 20 patients (90%) had a transferrin saturation greater than 36%. Hunter *et al.*, (1984a) found that levels of transferrin correlated well with the clinical course and survival, depressed levels of serum transferrin appeared to be a poor prognostic indicator. Low levels of serum transferrin were associated with death from sepsis. In this study, 6 out of 20 patients (30%) in phase 1 presented with low serum transferrin levels. The authors suggest that levels of transferrin in serum, together with its degree of saturation with iron and the development of transferrin receptors on cells contribute to the regulation of the balances between nutrition, inflammation or infection and the proliferation of various types of cells (Hunter *et al.*, 1984a). In a different study of 91 children with acute leukaemia, the patients were divided into 3 groups. Group 1 had low, group 2 medium and group 3 high serum iron levels. Of the 17 patients in group 1, 94% were alive 12 years after initiation of chemotherapy. In group 2, 41 patients (40%) died within 4 years and 75% within 8 years, the remaining 25% were alive at 12 years. Of 33 patients in group 3, 40% died within 2 years, 80% within 4 years and 100% within 7 years (Weinberg, 1986). The author suggested that the inability of patients of the latter group to lower their serum iron indicated a deficit in iron-withholding defence and/or an excessive amount of haemolysis.

All cells require iron for growth and metabolism to occur. This iron is obtained from a plasma-bound protein carrier, transferrin and is delivered to the cells via a specific cell

surface receptor (Barnett *et al.*, 1987). The transferrin receptor has been shown to be expressed on all proliferating normal and malignant cells (Hemmaplardh and Morgan, 1974). Habeshaw *et al.*, (1983) showed that the high grade lymphomas had a higher incidence of transferrin-receptor positive cells than low grade lymphomas and it was postulated that the transferrin receptor may be a way of predicting prognosis in non-Hodgkin's lymphomas. Subsequently, it has been demonstrated that the transferrin receptor is expressed with increased density in the majority of positive cases of acute leukaemia (Barnett *et al.*, 1987). The possibility that the expression of surface receptors for the iron-binding and iron-storage proteins might influence the progression of some forms of leukaemia was first proposed by De Sousa *et al.*, (1978). The authors postulated that the degree of saturation of serum transferrin could affect the accumulation of cells expressing receptors for transferrin and that a high transferrin saturation might be linked to the leukaemic process.

Iron chelators have been shown to inhibit growth of leukaemic cells *in vitro* (Foa *et al.*, 1986) and tumour growth *in vivo* (Sussman, 1989). Desferrioxamine has been used successfully in a 6-week-old infant with acute leukaemia who failed to achieve remission with chemotherapy (Estrov *et al.*, 1987). The probable mechanisms whereby high serum iron and high transferrin saturation could favour the growth of a transformed cell population include the provision of a nutrient indispensable for DNA synthesis (Foa *et al.*, 1986; Weinberg, 1986) and host immunoregulation (Brock, 1986; Weinberg, 1986).

Information regarding the host-bacterium interaction in infection is limited, particularly in the leukaemic patient and infection remains one of the major causes of morbidity and mortality (Bodey, 1986; Griffith *et al.*, 1989). In this investigation the antibody response during *P. aeruginosa*, *E. coli* and *K. pneumoniae* infection episodes was examined longitudinally. These organisms emerged as predominant representatives of bacteria, causing septicaemias in leukaemic patients. Such a study is difficult in clinical infection, as the response of patients is complicated by factors such as nutritional state, age, sex and the existence of other complicating diseases and therapy. Nevertheless, time coursing the development of antibodies in serum, following infection was considered a useful means of investigating the immune response in this group of patients.

Preliminary data confirmed previous reports (Cochrane, 1987) that the early systemic immune response involved predominantly IgG and IgM antibodies. The IgM response was found to peak and then decline rapidly, whereas the IgG response detected in the serum appeared to increase throughout the course of infection. However, all patients exhibited a poor antibody titre in response to infection, due to the immunosuppressive effect of the antineoplastic agents. The data presented are the first studies in which immunoblotting has been used to investigate the sequential antibody response to bacterial antigens during infection in leukaemic patients and to link this response to their iron-status.

The antibody response to *P. aeruginosa* was directed against the IRMPs and the major OMPs, particularly OprD, OprH and latter on in the infection episode OprL. The flagellar protein was strongly recognised by serum antibodies. This antigen has been shown to be highly immunogenic in other studies (Mutharia *et al.*, 1982; Anwar *et al.*, 1984; Ward *et al.*, 1988). Motility is thought to be an important virulence factor (Drake and Montie, 1988). Motile organisms rapidly disseminate from the original site of infection resulting in septicaemia (Anderson *et al.*, 1989) and antibodies which inhibit the functioning flagellum are likely to reduce the invasive capacity of the organism. The absence of flagella in the OM preparation obtained from patient PAVI may reflect shearing and loss of this antigen during sonication. The lack of recognition of IRMPs in the patient PAGF indicates that *P. aeruginosa* was either growing under iron rich conditions in the leukaemic host or that the IRMPs were not recognised by antibodies present in the serum early in the infection episode. However, an iron-rich environment in this patient is suggested by an initial high serum iron level, low serum transferrin level and 100% transferrin saturation. This indicates the presence of a pool of iron, bound non-specifically to other serum proteins, which is more accessible to invading bacteria (Brock, 1986).

The poor recognition of OprF was demonstrated, suggesting the protein is less antigenic than other OMPs or less accessible to the immune system. However, Lambert and Booth (1982) and Mutharia and Hancock (1983) using different techniques both demonstrated that the porin is located on the cell surface. A possible explanation is that the OMPs of *P. aeruginosa* used in these immunoblotting studies have been treated with SDS and 2-mercaptoethanol. Conformational epitopes in the antigens may have been affected by this

treatment (Mutharia and Hancock, 1985). Hence the low level of recognition to OprF in this study may be an artefact of the technique or may reflect the true response to this protein following onset of infection in the leukaemic patient. This poor reaction to OprF has been observed previously in burn wound infections (Ward, 1987).

Despite 50% of the *E. coli* septicemic patients exhibiting an antibody response to IRMPs, there was no recognition of such proteins in the remaining patients, similar to that observed previously. This indicates that *E. coli* may be growing under iron rich conditions in the host environment. From the iron study, these patients exhibited a high serum iron and low transferrin level, with transferrin saturation reaching 100%. This high percentage saturation of transferrin in patients with leukaemia raises the possibility that non-transferrin-bound, low-molecular-mass, serum iron complexes might be present in this disease. Such iron complexes can mediate tissue damage in several disease states by catalysing free radical reactions, such as the formation of highly reactive hydroxyl radical ($\cdot\text{OH}$) from superoxide (O_2^-) and hydrogen peroxide (H_2O_2 ; Gutteridge *et al.*, 1981). This is especially relevant because several of the drugs used to treat leukaemia, such as daunorubicin and adriamycin lead to increased generation of O_2^- and H_2O_2 *in vivo* (Halliwell *et al.*, 1988).

The finding that *E. coli* expresses porin proteins when grown under iron-depleted conditions *in vitro* and that these proteins are recognised by IgG antibodies in convalescent sera from infected patients, suggests that they are expressed *in vivo* in septicemia. These two peptidoglycan associated porin proteins known as OmpF and OmpC are immunologically related to each other (Lodge, 1987).

The main immune response for *K. pneumoniae* was directed against the IRMPs and the major protein bands 35.5 kDa and 39 kDa. In addition, an antigen with a molecular weight of 49 kDa was strongly recognised, although it was not evident on the coomassie blue-stained polyacrylamide gel (data not shown). Shand *et al.*, (1985) found that *K. pneumoniae* isolated directly from the urine of a patient showed a 48 kDa protein to be highly immunogenic, but it was undetected by coomassie brilliant blue staining. The leukaemic patients elicited a strong immune reaction to IRMPs, despite 100% transferrin saturation at onset of infection. However, both patients maintained the ability to reduce

serum iron and transferrin saturation during convalescence, which may explain the strong recognition of IRMPs. The lack of initial antibody response in the acute phase to the IRMPs for KPSW has been observed previously in *P. aeruginosa* chronic lung infection (Shand *et al.*, 1991). The possibility of a cross-reaction between sera from the patients and species of *Enterobacteriaceae* must also be considered. Shand *et al.*, (1985) suggest that it is probable that all 'normal' individuals have a low antibody titre against some OM antigens of the normal gut flora which are strongly detected by this system. The exact nature of the cross-reactions between the strains remains to be investigated with a more specific system, possibly with monoclonal antibodies against these antigens.

It has been reported that OMPs separated by SDS-PAGE and transferred to NC paper may contain co-migrating LPS (Poxton *et al.*, 1985). Probing replicate immunoblots of OMPs with patient's serum before and after absorption with purified LPS resulted in identical patterns and intensities of bands. The LPS was purified from cells cultivated *in vitro* because sufficient bacteria could not be obtained from the patient. It is possible that if the LPS from bacteria taken directly from the leukaemic host and those grown *in vitro* differ, absorption of the sera with the latter may not remove all the antibodies to the former. Nonetheless, the results suggested that the presence of LPS on the immunoblot did not contribute significantly to the bands observed.

The ability of these proteins to elicit early antibody formation indicates that they are accessible to the immune system, either by virtue of their surface location or following phagocytic processing. Sub-MIC levels of antibiotics may also affect envelope properties of bacteria such that OMPs become surface-exposed and thus available to interact with antibodies directed against them (Kadurugamuwa, 1985c). The high molecular weight IRMPs of several Gram-negative microorganisms, including *E.coli* (Griffiths *et al.*, 1983) were shown to be exposed on the bacterial surface using ¹²⁵I-lactoperoxidase labelling. Smith *et al.*, (1991) have shown that the 85 kDa IRMP of *P. aeruginosa* is surface-exposed and immunogenic using monoclonal antibodies, immunogold labelling and cell surface iodination. Protein D, OprF and OprG were also found to be exposed on the surface of *P. aeruginosa* by labelling with ¹²⁵I-lactoperoxidase (Lambert and Booth, 1982). Mutharia and Hancock (1983) using monoclonal antibodies to OprL found no fluorescence with intact

wild type cells cultivated in complex media, but did demonstrate a reaction with rough LPS-deficient mutant. Hence, they suggested that only strains lacking LPS O-side chains have OprL as a surface antigen, as observed for *P. aeruginosa* patient PAGF.

The antibody response to *P. aeruginosa* antigens has been investigated by other researchers in different types of infection, the results not always reflecting those observed with leukaemic patients. Serum from patients with urinary tract infection due to *P. aeruginosa* barely detected the IRMPs (similar to that found in some leukaemic hosts), whereas concentrated urine from the same patient clearly had antibodies to those proteins (Shand *et al.*, 1985). Additionally, proteins D, E, H and OprF and flagella antigen were recognised, a response closely resembling that of burn patients (Ward *et al.*, 1988) and that obtained in a rat model of chronic lung infection (Cochrane *et al.*, 1988). Sera from CF patients detected IRMPs, proteins D and OprL and showed a particularly strong reaction with OprF and OprG (Anwar *et al.*, 1984; Brown *et al.*, 1984; Cochrane *et al.*, 1988). In a study of 9 patients with CF the earliest antibody response to *P. aeruginosa* OMPs was directed against OprG, OprH and OprI (Shand *et al.*, 1991). The authors suggested that these proteins may be useful markers for the onset of infection. Additionally, it was found that antibodies to IRMPs did not appear in significantly high titres, early in the infection in any of the patients studied, which was considered an unexpected observation if it is assumed that the IRMPs are necessary for iron uptake and hence bacterial growth. In contrast, unpublished results in our laboratory suggest that the IRMPs are recognised during the initial stages of infection (Dr. J. West, personal communication). *P. aeruginosa* growing in the CF lung causes a chronic infection which over the years exposes many antigens to the immune system, so stimulating the production of high titres of antibodies to numerous bacterial components (Hoiby and Axelsen, 1973). *P. aeruginosa* in burn wounds and urinary tract infection initially produces an essentially acute infection involving the presence of a local immune response. In contrast, the leukaemic patient presents an acute and life-threatening septicaemic infection involving mobile cells which are highly invasive. Therefore, it seems that the nature (acute/chronic) and the site of infection as well as the host may influence the pattern of the immune response to OM antigens.

7. CONCLUDING REMARKS.

Septicaemia due to *P. aeruginosa* remains a major source of morbidity and mortality among patients with leukaemia. The proliferation of bacteria *in vivo* depends on two features that are not always easy to separate i.e. the ability to multiply *in vivo* and the ability to evade host defences.

Throughout this thesis the emphasis has been on the necessity of iron for bacterial growth and the ability of the host to sequester iron and make it unavailable. During clinical disease, host iron metabolism may alter and infection of a compromised patient (e.g leukaemic) is likely to differ significantly from that in a healthy host.

The results of this study have provided evidence for elevated serum iron levels and abnormalities in iron binding proteins in the leukaemic patient. Previous reports have found a reduction in the level of transferrin in leukaemia (Hunter *et al.*, 1984b) and in Kwashiorkor (McFarlane *et al.*, 1970). In both of these conditions there is an increased incidence of infection (Bullen and Griffiths, 1987). The possibility that iron may be made more readily available to some pathogens by an increase in the degree of saturation of transferrin, such as occurs in leukaemia, should be considered. A host environment rich in iron represented by high serum iron and high serum transferrin saturation, with low serum transferrin and total iron binding capacity might offer more favourable growth conditions to invading bacteria in the leukaemic patient. Therefore, in contrast to the surroundings encountered by bacterial pathogens in burn wounds, urinary tract infection or the CF patient, the leukaemic host might offer a significantly different environment to that noted previously. Future studies must, therefore, take into account the condition of the host and the effect that this may have on the environment encountered by potentially pathogenic bacteria *in vivo*.

Bacterial surface components expressed during infection play an important part in host-bacteria interactions. Identification of OMPs detected by antibodies in the sera of leukaemic patients provided evidence for important antigens during an infection episode. The ability of these proteins in *P. aeruginosa*, *E. coli* and *K. pneumoniae* to elicit early antibody

formation indicates that they are accessible to the immune system. These proteins may be useful markers for onset and identification of infection.

Furthermore, this study has identified additional proteins (for example OprG) which may be of value in vaccine preparations. Crucial to the future success of any immunotherapeutic strategies is the identification of key bacterial antigens which will stimulate prolonged levels of protective immunity against infection. An important factor in this respect is the realisation that bacteria may possess markedly different characteristics *in vivo* from those observed *in vitro*, some of which may correlate with increased virulence and pathogenicity of the organism (Brown and Williams, 1985b). Some critical antigenic determinants may be under-represented in conventional *in vitro* culture conditions. Hence, an increased understanding of bacterial properties in the *in vivo* environment is essential.

Immunosuppressed patients receiving antineoplastic chemotherapy are specifically susceptible to opportunistic infections (Ghannoum *et al.*, 1989). Treatment of microbial infections requires either discontinuing cancer chemotherapy or the use of drugs in combination, which can simultaneously treat both the neoplasm and the infection. The antineoplastic agent mitomycin C has been shown to exhibit antimicrobial activity against *P. aeruginosa* (Hamilton-Miller, 1984). Therefore, an organism not inhibited by this agent would have a definite advantage in establishing an infection.

The results presented here suggest that sub-MICs of mitomycin C caused perturbed expression of LPS in various strains of *P. aeruginosa* and preliminary evidence indicates that this would facilitate the actions of the host immune system and enhance clearance of the bacteria.

In the past the effect of different host environments on bacterial pathogens has been largely ignored. It does however, have important consequences not only for our understanding of bacterial pathogenicity and of diseases caused, but also for the design of measures used to prevent or treat infections. This situation is now changing rapidly and we are beginning to appreciate that specialised determinants are induced only when pathogens encounters its host (Griffiths, 1991).

Iron is now recognised as playing a vital role in infection. It constitutes a major environmental signal which coordinately regulates the expression of a number of virulence and metabolic genes. Progress in understanding the strategies used by pathogens for acquiring iron *in vivo* and their response to iron restriction is providing fresh insight into microbial pathogenicity. Iron is of such importance to the cell that many species of bacteria have developed both high and low affinity specific uptake systems. Here, it is shown that *P. aeruginosa* OprG is iron regulated *in vitro*. In contrast to the high molecular weight IRMPs, OprG is repressed under iron-limited conditions and expressed under iron-rich conditions.

Our increasing knowledge of the remarkable plasticity of the bacterial cell envelope may, however, also have important implications regarding sensitivity of pathogens to antimicrobial agents used in therapy. These studies show that the sensitivity of *P. aeruginosa* PAO1 to ciprofloxacin or gentamicin is dependent both on the level of available iron and on growth rate/phase. The increased expression of OprG noted with decreasing growth rate in the iron-limited chemostat could be either a direct influence of growth rate itself or a secondary phenomenon due to growth rate affecting the iron status of the bacteria.

Nosocomial infections of *P. aeruginosa* are a serious clinical problem, often due to its high intrinsic resistance to a number of antibiotics (Barriere, 1986). The process by which bacteria adapt to increased resistance to an antibiotic upon growth in the presence of that antibiotic is a poorly understood phenomenon. Interestingly, when cells of *P. aeruginosa* were trained to resist up to 30 X MIC of ciprofloxacin OprG expression was diminished.

In summary, the data presented in this thesis indicated that expression of OprG can be modulated by growth rate/phase, availability of iron and by the presence of ciprofloxacin in the growth medium. It is conceivable that OprG exhibits pore channel-forming activity.

8. REFERENCES

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