Response of Experimental *Staphylococcus epidermidis* Biofilms to Agents Used in Continuous Ambulatory Peritoneal Dialysis

by
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ABSTRACT

Patients with end-stage renal disease treated with continuous ambulatory peritoneal dialysis (CAPD) have a high incidence of peritonitis, caused principally by the skin commensal Staphylococcus epidermidis, a major limiting factor in the widespread acceptance of this form of renal replacement therapy. S. epidermidis reside on the surface of the permanent peritoneal catheter in a biofilm which may serve a protective function for the microorganism against the action of antimicrobials and host defenses and thus contribute to the development and recurrence of peritonitis. The main objective of this thesis was the experimental evaluation of agents prescribed to CAPD patients, primarily antibiotics for the treatment of infection, for their antimicrobial activity against experimental S. epidermidis biofilms.

Standardized preparations of S. epidermidis biofilms formed on various surfaces were studied in their intact state by a new technique, developed here, using a tetrazolium derivative as an indicator of metabolic activity. A large screen of commonly used antibiotics revealed rifampin to be exceptionally active against S. epidermidis biofilms. The superior activity of rifampin was exhaustively investigated with particular attention to the potential for synergy with several antibiotics. Unexpectedly antagonism was also demonstrated. These in vitro findings were confirmed in an experimental mouse model. Several other members of the rifamycin family indicated similarly potent antimicrobial effects against S. epidermidis biofilms. Disinfectants and drug additives to peritoneal dialysis (PD) solutions were similarly evaluated. Again, unexpected antagonism of rifampin activity was demonstrated by several additives, heparin and insulin amongst them. Fresh and spent PD solutions were found to modulate antibiotic action against S. epidermidis biofilms, with fresh PD solutions accentuating antimicrobial activity and spent fluids neutralizing it. The results obtained have direct implications to the clinical management of CAPD peritonitis.

RESUME

Les malades souffrant d'insuffisance rénale terminale et traités avec la dialyse péritonéale ambulatoire continue (DPAC) ont une incidence élevée de péritonites causées principalement par le Staphylococcus epidermidis, un commensal de la peau. Cette complication représente un facteur limitant majeur pour l'acceptation étendue de cette forme de traitement de remplacement rénal. S. epidermidis réside sur la surface du cathéter péritonéal permanent dans un biofilm qui peut servir une fonction protectrice envers le microorganisme contre l'action des antimicrobiaux et des défenses de l'hôte et ainsi contribue au développment et à la récurrence de la péritonite. Le but principal de cette thèse a été l'évaluation d'agents prescrits aux malades de DPAC, en premier lieu les antibiotiques pour le traitement d'infection, pour leur activité antimicrobienne contre des biofilms expérimentaux de S. epidermidis.

Des préparations standardisées de biofilms de S. epidermidis formés sur des surfaces variées ont été étudiées à l'état intact par une nouvelle technique, développée ici, utilisant un dérivé tétrazolium comme indicateur de l'activité métabolique. Un examen étendu d'antibiotiques fréquemment utilisés a révélé que la rifampine est exceptionnellement active contre les biofilms de S. epidermidis. L'activité supérieure de la rifampine a été investiguée en profondeur avec une attention toute particulière au potentiel de synergie avec plusieurs antibiotiques. De manière inattendue un antagonisme a aussi été démontré. Ces résultats in vitro ont été confirmés dans un modèle expérimental chez la souris. Plusieurs autres membres de la famille des rifamycines ont indiquées semblablement des effets antimicrobiens puissants contre les biofilms de S. epidermidis. Les désinfectants et les drogues ajoutées aux solutions de dialyse péritonéale (DP) ont été évalués pareillement. De nouveau, un antagonisme imprévu de l'activité de la rifampine a été démontré pour plusieurs additifs, l'héparine et l'insuline parmi eux. On a montré que les solutions de DP fraîches et drainées ont un effet modulateur sur l'action des antibiotiques contre les biofilms de S. epidermidis, les solutions de DP fraîches accentuant l'activité antimicrobienne et les liquides drainés la neutralisant. Les résultats obtenus ont des portées directes sur le maniement clinique des malades de DPAC.

For my parents,

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PREFACE

I developed the bacterial biofilm model, the mouse model of catheter-associated biofilm infection and the cytotoxicity assay of mouse peritoneal cells. I performed all of the procedures described here to the exception of the following: Dr. Francine Gervais contributed her assay of hydrogen peroxide production by mouse peritoneal macrophages; Dr. Barbara Gallimore contributed the data characterizing the mouse model of renal failure (Tables 18 and 19); and Gerry Bibeault contributed all routine hemograms of mice.

This work yielded a considerable amount of data, all of which has been or will soon be published. A list of the publications is given in Appendix 3. Many of the results recorded from this work are original findings, which are listed below (1-10).

1. A novel method for in situ bacterial biofilm investigation was developed. This method allowed the in vitro formation of standardized reproducible biofilms on plane or curved surfaces of Silastic or glass materials. The bacteria enmeshed within the exopolysaccharide matrix were assessed for metabolic activity using a tetrazolium derivative, TTC, and the reaction was interpreted as an indication of viability. This assay system permitted the evaluation of multiple test situations concurrently and obviated any requirements for specialized equipments or

materials. The technique of assessing metabolic function of microorganisms was also applied to determine the activity of catheter adherent biofilms recovered from patients and animals.

2. The aforementioned method was used primarily with standardized reproducible S. epidermidis biofilms of minimal density formed on glass microscope slides or Silastic CAPD catheter segments. The in vitro preparation, using a prolific slime producing strain of S. epidermidis, would yield uniform biofilms within 18 hours.

3. The superior antimicrobial efficacy of rifampin against S. epidermidis biofilms in vitro was discovered amongst an evaluation of 35 antibiotics. The most striking finding was the complete fluid phase sensitivity of this particular strain to all antibiotics screened, in marked contrast to the results of biofilm susceptibility. An evaluation of rifampin kinetics in vitro using peptone water and fresh peritoneal dialysis (PD) solution as the exposure media against S. epidermidis in the biofilm phase indicated a non-linear relationship with respect to concentration and exposure time. This contrasts with results observed for fluid phase S. epidermidis and demonstrates that similar microorganisms which exist in two distinct phases of growth do not share entirely identical mechanisms of antimicrobial killing.

4. The emergence of rifampin-resistant S. epidermidis was characterized in the biofilm phase when S. epidermidis biofilms were exposed to rifampin at concentrations up to

100 µg/ml in peptone water.

5. The ability to complete the partial killing effect of rifampin in peptone water by the separate combination of rifampin with cloxacillin, oxacillin and vancomycin was demonstrated in vitro against S. epidermidis biofilms. The emergence of rifampin resistance could also be prevented by changing the exposure media to fresh PD solution. This mutation of rifampin-resistant S. epidermidis was extensively assessed for fluid phase and biofilm phase characteristics and compared to the parent strain.

6. Under varying conditions, significant and complete antagonism of rifampin activity in vitro against S. epidermidis biofilms was revealed. Antimicrobial killing was completely abolished when using spent PD fluid as the exposure medium. In an effort to duplicate the effect of spent PD fluid, fresh PD solutions were modified by the addition of buffer or serum. Both modified fresh PD solutions antagonized rifampin activity to varying degrees, demonstrating a pH dependence and possible protein binding of the molecule. Additionally, several antibiotics including clindamycin, fusidic acid, gentamicin and tetracycline as well as several i.p. drug additives to PD solutions, heparin and insulin amongst them, completely antagonized the antimicrobial activity of rifampin against S. epidermidis biofilm preparations.

7. A comparative analysis of the antimicrobial activity of various preparations of rifampin, of two rifampin analogs and of the parent compound rifamycin SV against

S. epidermidis biofilms in vitro was established. It was shown that all these members of the rifamycin class of antibiotics share potent antimicrobial activity against S. epidermidis biofilms. Furthermore, it was also demonstrated that the rifampin analogs could effectively kill biofilms in spent PD fluid.

8. A range of antimicrobial activity against S. epidermidis biofilms was established for commercially available antiseptics/disinfectants as well as a new series of antimicrobial products (RenNew-P).

9. A mouse model of infection involving the i.p. implantation of catheter adherent S. epidermidis biofilms for the evaluation of host response and drug efficacy was developed. The outstanding antimicrobial activity of rifampin against S. epidermidis biofilms was demonstrated in this animal model.

10. A method for in vitro assessment of the cytotoxic effects of fresh PD solutions on resident and elicited populations of mouse peritoneal cells was developed. It was confirmed that PD solutions are highly cytotoxic to peritoneal cells, mostly resident populations, and that the degree of cytotoxicity correlated with the dextrose concentration of the PD solution. The assay was also used to assess the effect of common but variously administered non-antibiotic i.p. drug additives to PD solutions on cell viability. Most significant and detrimental effects were observed with phosphatidylcholine.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

BUN	Blood urea nitrogen
CAPD	Continuous ambulatory peritoneal dialysis
CFU	Colony forming unit
C-NS	Coagulase-negative staphylococci
CSF	Cerebrospinal fluid
°C	Degrees centigrade
DEAE-sepharose	Diethylaminoethyl-sepharose
dl	Deciliter
DNase	Deoxyribonuclease
ESRD	End-stage renal disease
FCS	Foetal calf serum
FMN	Flavin mononucleotide
g	Gram
g	Force of gravity
HCl	Hydrochloric acid
HEPES	(4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid)
H_2O_2	Hydrogen peroxide
hr	Hour
IgG	Immunoglobulin G
i.p.	Intraperitoneal
kg	Kilogram
MEM	Minimal essential medium, Eagle (modified), with Earle's salts and glutamine
mg	Milligram
min	Minute
MIC	Minimal inhibitory concentration
ml	Milliliter
mm	Millimeter
mM	Millimolar
mOsm/kg H_2O	Milliosmole per kilogram of water
µg	Microgram
µl	Microliter
µm	Micrometer
N	Normal
NAD	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide

Normal saline	Solution of sodium chloride, 9 g/liter
nm	Nanometer
nmole	Nanomole
PBS	Phosphate buffered saline
PD	Peritoneal dialysis
pH	Potenz hydrogen
PMN	Polymorphonuclear
PTFE	Polytetrafluoroethylene
PVC	Polyvinyl chloride
PVE	Prosthetic valve endocarditis
Redox	Reduction-oxidation
R-IM	Recovery-indicator medium
RR	Rifampin-resistant
RS	Rifampin-sensitive
S.	Staphylococcus
SD	Standard deviation
SEM	Scanning electron microscopy
sec	Second
TEM	Transmission electron microsocpy
TSB	Tryptone soya broth
TTC	Triphenyltetrazolium chloride
U	Unit
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1 CAPD and *Staphylococcus epidermidis* Infection

1.1.1 The Complication of *Staphylococcus epidermidis* Peritonitis

Continuous ambulatory peritoneal dialysis (CAPD) was introduced as an alternate form of dialysis therapy for patients with end-stage renal disease (ESRD) during the late 1970's (178,179). CAPD represents a manual form of peritoneal dialysis which requires a minimum of equipment and is usually performed by the patient at home. The procedure for adults involves the intraperitoneal (i.p.) instillation of 2-3 litres of dialysis solution by gravity via a permanent indwelling peritoneal catheter. The commercially available peritoneal dialysis solutions are hyperosmolar and acidic crystalloid solutions. They contain dextrose as the osmotic agent, lactate most commonly as the buffer and several electrolytes including sodium, magnesium, calcium and chloride. Heat sterilization of these solutions requires a reduction of pH by the addition of small amounts of hydrochloric acid to prevent caramelization of dextrose (188). These solutions are permitted to dwell intraperitoneally for a period of 4 to 8 hours to allow the diffusion of end-products of normal cell metabolism, most notably urea and creatinine from protein catabolism, into

the peritoneal cavity as well as fluid transfer according to osmotic forces (161). Following a clinically recommended dwell period, the peritoneal cavity is drained of fluid with the effluent being collected by gravity into an empty bag. Most patients perform 4 cycles per day, averaging 30 to 40 minutes to complete the procedure of dialysis exchange (60,160).

Physiologically, CAPD is the only maintenance dialysis method resembling normal renal function, in that it is performed continuously 24 hours a day, 7 days a week, 365 days a year. However, both the design of this form of dialysis therapy (presence of a permanent indwelling peritoneal catheter with a cutaneous exit site) and the procedure involved (repeated opening and closing of the circuitry at the time of exchange) require the utmost attention for scrupulous sterile technique. Failure of sterile practice often leads to infection at the cutaneous exit site, within the subcutaneous tunnel tract, and also within the peritoneal cavity itself, a much more serious complication. Despite recent advances in technology, peritonitis remains the major limitation of CAPD therapy for ESRD patients and is the leading cause of transfer to other forms of dialysis (28,40,86,91,102,191,214).

Peritonitis has been quoted as the "Achilles heel" of CAPD therapy, inasmuch as all patients are at risk of developing this complication, often within the first few months of dialysis (60,172,173). Although the incidence of peritonitis is variable between centers, an overall average

in Canada for the past 5 years is approximately 1.3 episodes per patient/year (116). In the majority of cases the pathogenesis of CAPD peritonitis remains unknown (91,182). The most frequent routes of peritoneal contamination include the lumen of the peritoneal catheter during dialysis exchanges and the subcutaneous tunnel tract around the catheter from cutaneous exit site infections (152,214). The identification of the microorganisms which are recovered from the peritoneal dialysis effluent during episodes of peritonitis has implicated these routes. The most prevalent causative microorganisms are consistent with the normal colonizing skin microflora (91,182,191,214). The most common pathogens associated with causing CAPD peritonitis are the Gram positive coagulase-negative staphylococci, and principally Staphylococcus epidermidis which accounts for 45% of all episodes (60).

Although mortality is rarely the end-result of CAPD associated bacterial peritonitis, morbidity can be very high (42,70,176). Most episodes are treatable while the patient remains ambulatory, with the relative severity of symptoms being dependent on the nature of the infectious pathogen. Despite the relatively mild symptomatology of S. epidermidis infections and the initial success of chemotherapeutic regimes, they are frequently relapsing in character (60,182).

Peritonitis is defined as an inflammation of the peritoneal membrane and is diagnosed clinically by a polymorphonuclear leukocyte count greater than 100 cells/mm^3

in the dialysis effluent in association with cloudy appearance and culture positive results. Symptomatic manifestations include abdominal tenderness, fever and general malaise, all of which would necessitate prompt initiation of therapy (60,116,160). When possible, the choice of a therapeutic regime is based initially on the Gram stain results of concentrated peritoneal effluents (60,216). Cases in which Gram stain identification by microscopy is not conclusive or may be absent entail the use of an aminoglycoside and either a first generation cephalosporin or vancomycin as initial antimicrobial therapy (116). However, the availability of numerous antimicrobials as well as differences in the etiology of peritonitis and susceptibility of microorganisms has led to a lack of uniformity in the literature for initial empiric recommendations (173).

Traditionally, the administration of antimicrobials has been i.p. via the inflowing fresh peritoneal dialysis solution. Some centers will perform peritoneal lavage (3 quick successive exchanges) to clear the peritoneal cavity of cellular debris and fibrin as well as to relieve pain, prior to initiating therapy (60). Peritonitis will frequently lead to fibrin clot formation within the peritoneal cavity, resulting in possible catheter obstruction. To prevent this heparin is commonly added to all inflowing dialysis solutions until the infection has been resolved and the peritoneal effluent presents no evidence of fibrin clots (116,160).

The duration of antimicrobial therapy is controversial, being dependent on the microorganisms involved and their degree of susceptibility. In general, most centers will treat uncomplicated episodes of peritonitis for 10 to 14 days, although prolongation of therapy has recently been suggested to prevent relapsing infections particularly when S. epidermidis has been isolated (86,87). The current management strategy for persisting peritonitis with Gram positive microorganisms indicates the discontinuation of cephalosporins and the addition of rifampin to vancomycin therapy should improvement fail to occur by 96 hours of instituting the initial therapeutic regimen. Relapsing or recurrent peritonitis is defined as a reinfection of the peritoneum by the same microorganism within 4 weeks of arresting therapy. The empiric recommendation upon identification of S. epidermidis includes an intensified combination of vancomycin and rifampin for a period of 2 to 4 weeks (60,116). Failure to resolve any episode of peritonitis through the aforementioned regimes, requires a discontinuation of CAPD therapy and catheter removal (160).

1.1.2 Host defenses of the peritoneal cavity

The peritoneal cavity normally contains 50 to 100 ml of clear fluid which serves the dual purpose of lubricating the visceral organs and immunologically providing a first line of defense against intruding pathogens (118). The resident population of this fluid approximates 3000 cells/mm^3 and consists predominantly of macrophages (117). During CAPD the peritoneal cavity continuously encompasses large volumes of dialysis solution which reduces the concentration of the resident cellular population approximately tenfold (118). Both the large volume used and the non-physiological nature (hyperosmolarity, acidity) of commercially available peritoneal dialysis solutions have been shown to adversely affect the local components of peritoneal host defense (2,79,101,190,215).

It has become common practice to use fresh dialysis solutions as a vehicle for the administration of various drugs aimed at local or distant sites of action. Antibiotics are therapeutically administered i.p. during peritonitis, but more frequently is the addition of heparin to prevent fibrin clot formation during inflammatory states and blood clot formation immediately following surgical placement of the catheter; and insulin for convenience as well as improved control of blood glucose levels in diabetics undergoing CAPD (4,57,71,76,104). More recently drug additives have included those which increase

ultrafiltration rates across the peritoneal membrane (furosemide, verapamil, phosphatidylcholine) (58,97,165), have thrombolytic properties (streptokinase, urokinase) (162,175,225), chelate specific ions (desferrioxamine) (16,105,204,205), and vitamins (calcitriol) (54). All of these therapeutic agents either alone or in combination(s), may adversely affect local host defenses either through cell lysis or disruption of phagocytic functions. Furthermore, it is unknown whether the administration of these non-antibiotic drug additives during episodes of peritonitis, can interfere with the efficacy of current antimicrobial regimes.

The introduction of pathogens in the peritoneal cavity evokes local host defense mechanisms which include cell mediated and humoral immune responses (118,215). A rapid change in cell population ensues when peritoneal macrophages can no longer control the growth of intruding pathogens. Polymorphonuclear (PMN) leukocytes, attracted by chemotactic stimuli, enter the peritoneal cavity from the circulation by diapedesis and are recognized as a cardinal sign of peritonitis (173).

The process of phagocytosis of microbes is dependent on several factors, which during CAPD may be adversely affected by the continuous presence and regular cycling of dialysis solutions within the peritoneal cavity (80,172). Peritoneal dialysis effluent from CAPD patients has been shown to contain only 1% of the normal levels of opsonic molecules regularly found within the peritoneal cavity (118,173).

Indeed, the level of IgG within the peritoneal fluid correlates with the ability of an elicited population of PMN leukocytes to phagocytose bacteria, especially S. epidermidis. It was recently demonstrated that 40% of CAPD patients have consistently low concentrations of IgG within their dialysis effluent, and presented with a sevenfold greater incidence of S. epidermidis peritonitis (117). Furthermore, preliminary studies of passive immunization of these patients with i.p. infusion of gamma globulins markedly reduced their rates of peritonitis (117). These findings suggest that the relative concentration of opsonins at the site of pathogenic invasion may be critical in determining the degree to which infection in the peritoneal cavity advances (3,63).

Both macrophage and PMN leukocyte functions against S. epidermidis are adversely affected by fresh dialysis solution and dialysis effluent, uninfected and infected; due to the acidic pH, hyperosmolarity and a lack of opsonic molecules in the former and a deficiency of opsonins and the presence of a recently discovered inhibitor molecule for the latter (80,101). This as yet unidentified inhibitor molecule interferes with the intracellular killing of bacteria by PMN leukocytes. This finding may explain why, in the presence of sufficient opsonins, S. epidermidis are not lysed within the phagolysosomes and thus are able to remain sequestered within the inflammatory cells (25,213,217). Indeed, viable S. epidermidis have been located primarily within macrophages and PMN leukocytes

recovered from CAPD patient effluents. Despite the low virulence of S. epidermidis, infections caused by this microorganism are often difficult to eradicate and frequently recur after apparently adequate antimicrobial therapy (14). It is significant that current initial management regimes for S. epidermidis peritonitis do not include a class of leukocyte-penetrating antibiotics such as clindamycin or rifampin (60,116). The microbial parasitism of S. epidermidis by intraleukocyte sequestration may be one explanation for relapsing peritonitis (172).

The uremic state is characterized by a number of disturbances, including impaired immune responses which are characteristically difficult to demonstrate. More prominent manifestations of chronic uremia include the retention of nitrogenous products, anemia, bone disease, enzyme abnormalities, growth retardation in children, fluid and electrolyte imbalances, and a number of various metabolic and hormonal disorders (60).

The immunosuppression associated with chronic renal failure may also be a factor contributing to the pathogenesis of CAPD peritonitis (38,150). Although much of the information regarding immunodeficiences in chronic renal failure has been derived from research in hemodialysis patients, a number of abnormalities, particularly an enhanced suppressor cell activity, have been described for this patient population (60). However, these physiological imbalances may arise in part from the hemodialysis procedure itself. As yet, a direct correlation between the influence

of chronic uremia and dialysis upon the immune response of the host to infection remains to be established (37,155,156). In general, bacterial infections occur more readily and with greater severity in dialysis patients as compared to non-uremic counterparts (60). The limited clinical investigations of chronic renal failure have not consistently identified major immunological deficiencies which may predispose the dialysis patient population to infection. Recent animal studies have provided evidence for an increased susceptibility to defined challenge with S. epidermidis in progressive chronic renal failure models (78). Thus, despite conflicting data, renal failure related infections due to compromised host defenses must be considered in the pathogenesis of CAPD peritonitis.

1.1.3 CAPD Catheters and Infection Prophylaxis

In 1947 Palmer began to develop a catheter which could remain permanently within the peritoneal cavity for the purpose of chronic peritoneal dialysis. Soon after, the first permanent silicone rubber (Silastic) based catheter was produced (59,160). It was designed with an exit site seal to prevent fluid leakage from the peritoneal cavity and for placement with a long subcutaneous track so as to minimize catheter movement. By 1968 Tenckhoff had modified the Palmer catheter, making it bacteriologically safe and thus available for chronic peritoneal dialysis (207). Minor complications of catheter dislodgement and obstruction prompted Oreopoulos, Goldberg and Hill to further modify the basic Tenckhoff design, through improving its stabilization within the pelvis and preventing the omentum from catheter attachment (163). Indeed, the practical application of maintaining long term access to the peritoneal cavity decreased the incidence of tunnel infections which often plagued the early catheters for chronic dialysis (160). However, infections do remain associated with currently used catheters and often complicate this form of dialysis.

Periluminal catheter infections are enhanced by dialysate leakage, loosening of the catheter, poor aseptic procedures and possible breakdown of cutaneous tissue due to the frequent administration of topical antiseptics (60). Exit site infections which go unchecked will often progress

along the subcutaneous tunnel tract to the peritoneal cavity. Much interest has therefore centered around the use of disinfectants applied at the peritoneal catheter external connection and exit skin site as an adjunct to the prevention of CAPD peritonitis. Other efforts to diminish the risk of peritonitis through accidental intraluminal contamination at the time of exchanges include: the Dupont box for automatic thermal sealing of catheter connections (99); the filtering of inflowing fresh peritoneal dialysis fluids (6,229); and the use of UV light as a germicidal system (1). The recent introduction of new connectology with antimicrobials placed directly in the lumen of the external tubing system offers a particularly attractive approach (26,27,136).

Morphological examination of indwelling peritoneal catheters, following several months after placement, has demonstrated that all intraabdominal sections on both the luminal and extraluminal surfaces are covered with an adherent bacterial biofilm containing S. epidermidis predominantly (52,172). The pathogenic significance of bacterial biofilms is presently unknown, although they may be serving as a bacterial reservoir which under conditions of compromised immune defenses may yield clinical expression of peritonitis. The development of disinfectants/antiseptics for i.p. instillation has particular appeal for the destruction of catheter adherent biofilms (Appendix 3.A.2).

1.2 Coagulase-Negative Staphylococci (C-NS)

1.2.1 General Class Characteristics of C-NS

There are presently 24 species of bacteria classified under the genus of Staphylococcus. These microorganisms are non-motile, facultatively anaerobic, Gram positive and possess catalase (88). The majority of the staphylococcal species are deficient in the production of coagulase and as such are grouped as coagulase-negative staphylococci (C-NS) (100). The cocci divide in multiple planes to produce clusters. The colonial appearance of C-NS when established on solid nutrient media are characteristically of white, smooth surfaced, circular colonies (114).

The surface of C-NS is comprised of an inner cytoplasmic membrane, a rigid cell wall, a capsule and variably an outer slime layer (46). The cytoplasmic membrane is a phospholipid bilayer which contains various proteins, enzymes and permeases. The cell wall is relatively thick and contains as major components peptidoglycan and teichoic acid, comprising 50% and 30 to 40% of the weight, respectively. Teichuronic acids, polysaccharides and various proteins complete the cell wall structure (50,228). The cell capsule and slime layer may share polymers which are composed primarily of polysaccharides (50). The cell capsule constitutes a polyanionic glycoprotein envelope of known structure firmly adherent to the cell wall and extending a distinct distance

from the surface. Slime, although ill defined, is a complex heterogenous mixture which when separated by DEAE-sepharose columns yields 27% protein and 40% carbohydrate predominating in derivatives of galactose, mannose and glucuronic acid (171,228). In contrast to the cell capsule, slime has no defined margin of growth and may be loosely associated with the cell surface (50,171).

Symbiosis is the close association of two organisms to their mutual benefit (75). Commensalism exists when the relationship is beneficial to one organism without effect on the other, much like staphylococci on the human skin surface. Recently, the delineation between commensalism and pathogenicity has become complicated by the everincreasing occurrence of bacterial infections which arise when microorganisms are displaced from their common environment (staphylococci on the skin) and introduced into a compromised system (peritoneal cavity of a patient receiving CAPD therapy) of the same host (9).

Staphylococcus epidermidis, a C-NS, is the most prevalent microorganism to colonize the human skin and mucous membranes, constituting 65-90% of all staphylococci (19). The natural resistance of C-NS to drying, salt, temperature, sunlight and various chemical agents makes them ideally suited for skin colonization as well as enhancing their survival on inanimate surfaces (100). Previously recognized as a frequent contaminant of blood cultures and a microorganism of minor pathogenic significance, S. epidermidis is now recognized to have significant

pathogenic potential and is known to cause infections associated with foreign implanted bodies and in immunocompromised hosts (19,50,134).

In 1972, Bayston and Penny proposed that a selective virulence of S. epidermidis exists when it first colonizes foreign bodies (13). They observed that strains of C-NS which caused cerebrospinal fluid (CSF) shunt infections in vivo would form adherent mucoid deposits on similar CSF shunts in vitro. Indeed, more recent studies have suggested that slime producing strains of S. epidermidis have a greater virulence when foreign bodies are present (33,50). Furthermore, slime producing strains of S. epidermidis are more frequently associated with symptomatic infections than non-slime producers thus further implicating slime as a virulent entity (31,64,111). These particular strains are also known to produce several exotoxins including δ-hemolysin, DNase and fibrinolysin. This is thought to contribute to the pathogenicity of these microorganisms and their ability to promote virulence (81). The transition between harmless colonization and clinical infection by C-NS is clearly complex, and seen to be dependent upon a combination of virulence factors as well as the current state of host defense mechanisms at the site of pathogen intrusion (80).

1.2.2 Adherence Properties of C-NS

Most bacterial species are known to exist in two distinct phases: a fluid phase in which microorganisms are independent and when tested possess the potential for mobility, and a fixed phase where the microorganisms are adherent to surfaces and organized in a matrix (43,44,45,47). This adherent mode of growth is representative of all but a small fraction of bacteria in natural environments (24,45). These microorganisms can colonize both animate and inanimate surfaces and proceed to grow in the fixed phase producing an extracellular glycocalyx material or slime-like fraction (46,50,94,95,171). This structured form of bacterial colonization is referred to as a biofilm.

Bacterial biofilms are surface adherent microbes forming colonies within an exopolysaccharide matrix (46). The most visible example is dental plaque occurring on teeth which is an adherent biofilm of mouth microorganisms (82,92). Bacterial biofilms can be produced in vitro with various strains of Streptococcus mutans and C-NS, which commonly demonstrate a similar mode of adherence followed by the production of a slime layer of predominantly polysaccharide material (32,153).

Prosthetic material implanted by surgical procedures is a factor known to make local tissues susceptible to both immediate and delayed infections (197). The literature

indicates that bacterial adhesion to biomedical polymers may be an essential step preceding the pathogenic state (19,50). Of the recognized species of C-NS, S. epidermidis and S. saprophyticus are two clearly considered as true pathogens (100). S. epidermidis infections are unusual in that they are most often associated with implanted medical devices and clinically reflect a high degree of morbidity and a variable incidence of mortality (134). The extensive list of medical implants/prostheses which are associated with clinical infections and presumed to follow bacterial adherence include: prosthetic heart valves (220), cardiac pacemakers (143,144), vascular prostheses (17,115,185), intravascular catheters (142,169,200,206,209), ventricular shunts (12,13) orthopedic hardware and prosthetic joints (93), bladder catheters (49,129,157,168), ocular prosthetic devices (201), intrauterine contraceptive devices (140), sutures (36,96) and peritoneal dialysis catheters (51,145,172).

Bacterial biofilm development on biomedical implants or tissues involves a series of sequential processes (31,45,109). Microorganisms are transported to within range of the biomaterial surface by hydrodynamic forces (50). Other physical forces, both attractive and repulsive, then develop between the bacteria, the fluid medium and the inanimate surface proper. They occur within approximately 15 nm from the surface and include hydrogen bonding, ion-pair formation, dipole-dipole interaction, van der Waal's effect, surface charge, surface tension and

hydrophobicity (50,95,166). This initial adsorption will remain reversible until attractive forces can maintain the microorganisms within sufficient proximity to ensure permanent adhesion (46,95,109). Specific molecules or foot-like organelles arising from the cell membrane will then bridge the intervening space (approximately 1 nm) and thereby initiate chemical bonding (46,95). Once adherent to the surface proper cell replication can proceed, first forming aggregates and then colonies of bacteria (62,109). The community production of exopolysaccharide material will encase the microorganisms and interact with host fibrin which becomes incorporated within the biofilm structure (50,95).

The affinity of bacteria to biomaterials is influenced by multiple interrelating factors (31). These include the physical and biochemical characteristics of both the pathogen and the implanted material, as well as the duration of exposure to the surface proper and the nature of the surrounding milieu (36,50,107).

C-NS have been shown to develop and proliferate in biofilms on the luminal and extraluminal surfaces of intravenous catheters in the absence of any externally supplied nutrients (133). A definite correlation was demonstrated between the degree of slime production, the increase in number of adherent C-NS to surfaces and the decrease in number of such microorganisms which existed in the fluid phase (170). Morphological examination of these catheters by scanning electron microscopy (SEM) showed

bacterial cells producing a single layered colony which was succeeded by multiple layers as the incubation period was prolonged. No evidence of slime production was apparent until 12 hours of incubation (170). The initial phase of adherence is primarily determined by hydrophobicity and is independent of slime production (50). The amount of polysaccharide material produced by the abovementioned strain of C-NS was shown to increase proportionally with incubation time following 12 hours, growing 120 μm thick within 24-48 hours (170). It was noted that surface irregularities (chips, crack-like defects, fissures, depressions, scrapings, scratches and cavities) were frequently detected by SEM in segments of intravenous catheters (132). These minor imperfections in surface structure provided preferential niches for early attachment of C-NS (50,133). However, smooth surfaces became homogeneously colonized with prolonged incubation and/or larger inoculum. After 48 hours of incubation SEM clearly demonstrated zones of erosion surrounding the adherent colonies, indicating the possible utilization of catheter material as nutrients to support bacterial growth (170). This would explain the remarkable proliferation of C-NS in vitro without the supplementation of growth media and is suggestive of the events which develop in vivo.

Pascual et al suggested that interfering with the process of hydrophobic bonding of bacteria to surfaces would prevent the colonization of medical implants and thus related infections (166). According to this author and

others, the adherence of C-NS to Teflon-based materials is directly related to bacterial hydrophobicity (7,50). This mechanism of adherence can be strongly inhibited but not completely abolished by preincubation of either catheter segments or bacteria in serum components. This ability to modify the adherence of microorganisms in the presence of serum proteins would suggest that a positive electric charge on biomaterials is regarded as an important factor promoting C-NS adherence (166). In contrast, other proteins may be involved in the process of catheter colonization by enhancing bacterial adherence. Fibronectin is an adhesive glycoprotein which acts as a bridge between polymers, such as collagen, as well as a coating to bacteria. The Russell group noted that fibronectin would adhere to polyvinyl chloride (PVC) based catheters and significantly increase the adherence of C-NS (192). The group of Hogt suggested that the surface associated proteins of bacteria, possibly specific attachment receptors, were also important for the process of surface colonization. They demonstrated that proteolytic enzyme treatment could decrease the ability of C-NS to adhere to fluorocarbon based materials (108).

The physicochemical properties of implanted biomaterials is also a critical determinant of bacterial attachment. Schmitt et al and Sugarman et al using slime producing strains of S. epidermidis and Enterobacteriaceae respectively, demonstrated that these two different microorganisms would adhere to Dacron with 10 to 100 times greater affinity than to polytetrafluoroethylene (PTFE)

(194,203). Gross differences in surface characteristics between these two materials may account for some variation in bacterial affinity. PTFE is relatively non-porous in appearance which contrasts markedly with the multi-filamentous nature of Dacron. Chemically, PTFE displays more hydrophobic properties than Dacron and is therefore less likely to form bonds with bacteria, such as C-NS, in which the cell walls are hydrophobic (194). The group of Sheth also demonstrated variations in bacterial affinity in relation to catheter composition. They showed that C-NS would adhere to and colonize PVC intravascular catheters preferentially over Teflon based catheters (198,199). These studies indicate that the type of implanted biomaterial may be a critical determinant of both the initiation and development of bacterial colonization of medical devices (50).

1.2.3 Effects of C-NS Slime on Host Defense and Antimicrobial Susceptibility

Foreign body infections associated with bacterial biofilms can be difficult to eradicate and commonly require the removal of the biomaterial in question (94,96,145). The protective nature of the exopolysaccharide matrix in which bacteria are sequestered can interfere with normal host defense mechanisms and alter the efficacy of various antimicrobial agents (24,46,81,171,172). As such, the slime-like adhesive of biofilm producing microorganisms is a proposed virulence factor in conjunction with persistent S. epidermidis biofilm infections (35,50).

The actions of a functional immune system are often impeded in the presence of medical implanted devices (230). Foreign bodies are non-vascularized entities and are therefore barriers to leukocyte penetration (100). Additionally, the microenvironment surrounding these biomaterials appears to adversely affect opsonization, phagocytosis and bactericidal activity of both macrophages and PMN leukocytes (145). The host response to chemotactic stimuli from pathogenic microorganisms existing in a biofilm is limited because of the physicochemical properties of the exopolysaccharide matrix (50). C-NS adherent to biomedical implants tend to be more resistant to surface phagocytosis than S. aureus or Pseudomonas aeruginosa which also colonize inanimate surfaces in biofilms and are classified as more

virulent microorganisms (80). Opsonization of S. epidermidis is essential for phagocytosis, and both immune processes occur readily when the microorganism exists in the fluid phase (81). However, developing colonies which have yet to produce slime are commonly opsonized but are characteristically not phagocytosed by macrophages or PMN leukocytes because they exist in large adherent clusters. Likewise, once slime production has surrounded the colonies of adherent bacteria, antibodies are prevented from penetrating the biofilm: presumably as a result of surface charge characteristics and/or physical obstruction (171).

Slime, as a separate entity, has demonstrated antagonistic effects towards the cellular components of the immune system. The exposure of neutrophils to crude preparations of this heterogenous material clearly alters normal functions (chemotaxis, superoxide generation, adherence, degranulation, phagocytosis) without an apparent effect on cell viability (80,159). The ability of slime to either inhibit PMN leukocyte degranulation of intracellular lysozymes or generation of reactive oxygen metabolites, or to cause inefficient premature discharge of these cidal contents may explain the lack of bactericidal activity against phagocytosed fluid phase S. epidermidis (113). This finding is consistent with some of the clinical observations of recurrent peritonitis in CAPD (81). Harvested peritoneal PMN leukocytes from these patients frequently demonstrate the ability to sequester viable C-NS following apparently adequate antibiotherapy (217). Slime has also been shown to

reduce the proliferative response of T and B lymphocytes to polyclonal stimulators, thus suggesting that the specific immune responsive cells may also be compromised by biofilm associated infections in vivo (80,89,90).

Bayston and Penny noted that CSF ventricular shunts removed from patients prone to recurrent infections were colonized with a bacterial-laden mucoid substance (13). They had been unsuccessful at clearing this early description of a biofilm with recommended therapies, even when the antimicrobials were administered directly into the colonized valve chamber (12). Catheters which are removed from patients because of recurrent S. epidermidis septicemia following apparently adequate antibiotherapy commonly reveal bacterial biofilms (up to 160 μm thick) adherent to the surface (171).

Bacterial biofilms function as ion-exchange resins (45). Their near infinite surface area and polyanionic nature can entrap nutrients from surrounding milieus to enhance bacterial growth (50). However this complex structure is also functioning as a barrier, to limit the passage of specific molecules (antibodies and antibiotics) according to their affinity and theoretical dissociation constants (50). This suggests that once an antimicrobial agent has exceeded the binding capacity of the glycocalyx structure, the molecule in question will eventually penetrate to the bacterial cell. In vivo observations of a low but clinically significant resistance of biofilm associated Pseudomonas aeruginosa to carbenicillin would

tend to support this concept (46).

The efficacy of current therapeutic regimes for C-NS infections are frequently not in concordance with the values predicted from laboratory antimicrobial susceptibility tests (45,100,134,171). Routine evaluation of biochemical and antimicrobial susceptibility profiles of clinical isolates involves multiple subcultures of the microorganisms in vitro. During the processing of these isolates, a microorganism may lose some of its in vivo characteristics (24,45). Furthermore, conventional microbiological testing is directed against fluid phase microorganisms and not against intact, adherent populations of bacteria (44). Thus, antimicrobials which are administered to eradicate sequestered infections should be screened for their ability to penetrate the extensive exopolysaccharide matrices of adherent bacterial biofilms (13,46).

The microorganisms associated with urinary tract and pulmonary infections are known to progress while existing in biofilms (41). Individual microbial cells or clusters are often sloughed from the biofilm matrix into the surrounding fluid milieu to initiate new colonies or cause general sepsis (43). Clinical isolates from urine or sputum which are subjected to conventional antimicrobial susceptibility testing show that low doses of strain specific antibiotics will kill these detached microorganisms and render the urine or sputum culture negative (43). However, the increased incidence of recurrent infections suggests that persistent biofilm encased microorganisms remain protected from

standard therapies. Indeed, biofilm embedded bacteria have demonstrated a 20 to 50 fold greater resistance to antimicrobial action over their fluid phase counterparts (109,130,200). The clinical urgency in choosing the correct therapeutic regime when biofilms are suspected as the source of implant-associated infections is indicated by the striking 70 to 80% early mortality rate for patients who develop prosthetic valve endocarditis, an infection frequently associated with C-NS (19,134,220).

1.2.4 C-NS: Potentially Serious Nosocomial Pathogens

Coagulase-negative staphylococci and S. epidermidis in particular, are recognized as significant nosocomial pathogens (100,177). Virtually all S. epidermidis infections, except for native valve endocarditis, are hospital acquired (19,64). The incidence of S. epidermidis surgical infections has increased in parallel with increased use of medical implants and prosthetic devices (64,100). These infections are undoubtedly arising from contamination by skin commensals although their origin, be it patient or hospital personnel derived, remains uncertain. One case of C-NS transmission, as demonstrated by plasmid profiling and DNA hybridization, has been documented between a surgeon and patient (100). If S. epidermidis infections are initiated by transmission via physical contacts between hospital personnel, documenting a chain of transmission would prove difficult due to the presence of multiple strains (>10) on a single individual (110).

The predominance of staphylococci as the causative pathogen of early prosthetic valve endocarditis (PVE) was recognised in the 1960's (220). The empiric recommendation of antistaphylococcal prophylaxis diminished the incidence of PVE. However, the efficacy of prophylaxis was compromised in the 1970's by the appearance of resistant staphylococci to commonly used prophylactic agents, notably methicillin (19,220). The pathogenicity and tenacity of

C-NS have steadily increased into the 1980's as these microorganisms have continued to develop resistance to a wide range of antimicrobial agents (31,34,50,64). This ability to accumulate antibiotic resistance may be transmitted by plasmid vector to more aggressive species (S. aureus) of the same host and may spread with every staff-patient contact in a hospital (81,154). Althcugh the conjugative mechanism of these plasmids is unknown, it would appear to require cell to cell contact (154).

This decade has been witness to an increased frequency of bacteremia associated with medical implanted devices (31). Explanations of such include a greater recognition of C-NS as a pathogen and not mere dismissal as contaminant. The increased number of invasive procedures performed as well as recommendations for long courses of broad-spectrum antibiotics which may have selected multi-resistant strains are other factors to be considered (64,139). Approximately 45% of hospital acquired infections are associated with implants or medical devices and consist of a case-to-fatality ratio ranging between 5 and 60% (50).

C-NS infections associated with indwelling foreign bodies are frequently indolent and thus devoid of clinical signs and symptoms (62,134,145,194). These pathogens are commonly associated with foreign body implants where they exist within surface adherent biofilms. Resulting infections are referred to as cryptic or biofilm associated and are constituting an increasingly important proportion of infectious diseases within developed countries (31). They

are often restricted to a single affected area and are seldomly characterized by a rapid bacteremic dissemination. Whether acute or chronic, these infections tend to persist despite an effective host defense system and intensive chemotherapeutic regimes, until the foreign body is removed (19,206). The nature of C-NS infections is often occult, as they may remain dormant for extended periods of time (weeks to years) before causing local or systemic signs and symptoms (19,62,96,115). Single bacteria or cohesive aggregates may randomly disseminate from the protective biofilm to cause general sepsis and/or further systemic disease by colonizing other tissues (43,92,115). The microorganisms which do disseminate are not necessarily representative of types, number or pathogenicity of bacteria remaining within the adherent biofilm and may therefore provide misleading antimicrobial susceptibility results (92). While fluid phase C-NS can be controlled with the use of specific antibodies and antibiotics, biofilm associated infections persist in patients with very high levels of specific antibodies in whom antibiotics proved ineffective.

1.2.5 CAPD Peritonitis and C-NS

As CAPD has become an established treatment worldwide for some 25,000 currently registered patients with chronic renal failure, the incidence of peritonitis has remained the major complication of this form of renal replacement therapy (172). Peritoneal dialysis patients demonstrate general immunosuppression. Most recognized is the frequent dilution of immune constituents and the cytotoxicity of peritoneal dialysis solutions as well as the intermittent drainage of the dialysis effluent (60,118). S. epidermidis is the most frequent C-NS species recovered during the episodes of CAPD peritonitis. The source of these pathogens is thought to arise from the colonizing microflora of the patient skin (172,197). Contamination of the peritoneal cavity is believed to occur via the dialysis solution delivery system during fluid exchanges and by progressing along the subcutaneous tunnel tract from the catheter exit site (81).

Adherent bacterial biofilms are currently considered as contributory factors in the development of medical implant-associated infections, including CAPD peritonitis (50,109). However, limited evidence has been presented to establish a causal relationship between microbial colonization of the intraabdominal segment of the peritoneal catheter and the development of CAPD-associated peritonitis (145,219). In 1981 Schuenemann et al described adherent bacterial cells enmeshed within a fibrin structure on the

indwelling extraluminal surface of Tenckhoff catheters (quoted in 109). They associated the presence of the persistent microorganisms with the occurrence of relapsing episodes of peritonitis. The group of Dasgupta and others have presented electron micrograph evidence of biofilm adherent bacterial colonies on both the extra- and intraluminal surfaces of Tenckhoff catheters which had been indwelling within the peritoneal cavity for periods of at least 3 months (51,52,53,172). The catheters were removed from CAPD patients for one of two reasons: recurrent episodes of peritonitis and successful renal transplantation. The results indicated that regardless of the clinical reasons for catheter removal, bacterial biofilms were uniformly observed (53). SEM also revealed a variety of morphotypes adherent to the surfaces, occasionally constituting mixed populations of gram positive cocci, gram negative rods and fungi, but more predominantly S. epidermidis (51).

None of the studies currently examining bacterial biofilms in vivo could demonstrate whether colonization of indwelling peritoneal catheters would progress to infectious episodes of peritonitis. The observations that catheters from uninfected asymptomatic patients are routinely colonized may suggest that peritoneal immune defenses are crucial in determining the pathogenicity of C-NS. These observations also reinforce the concept of possible latent infections, as biofilms are reservoirs of viable microorganisms which may lay dormant for extended periods

(51). Nonetheless, biofilms produced by C-NS function to protect the embedded microorganisms from host defenses and antimicrobial action and thus remain a possible explanation for recurrent CAPD peritonitis (124,182).

1.3 Bacterial Biofilms

1.3.1 Current Methods of Study of Bacterial Biofilms

Bacteria which are sequestered within a surface adherent biofilm may be overlooked by standard microbiological culture techniques. As such, various methods have been developed to investigate the qualitative and quantitative characteristics of bacterial biofilms in vivo and in vitro. Current techniques include: scanning and transmission electron microscopy (SEM/TEM), epifluorescent and bright field light microscopy, radioisotope labelling, spectrophotometry, specimen streaking onto sclid growth media, specimen flushing with fluid culture media and mechanical disruption. To varying degrees, these procedures are capable of assessing: the morphology and bacterial cell density of biofilms; the adherence properties as well as slime producing capacity which are characteristic of specific strains of microorganisms; and the metabolic activity of both fluid phase and biofilm state bacteria.

Illustrative electron micrograph work by Peters et al and Costerton et al amongst others have indicated the progressive development of bacterial biofilms in vitro and its presence on numerous medical implants/prosthetic devices (45,46,92,93,94,169,199). Although the dehydration of the exopolysaccharide structure of biofilms is inevitable during the processing for SEM/TEM, the techniques provide excellent

morphological definition of specimens in situ. Limitations of SEM/TEM are the requirement that microorganisms be killed in the preparatory process, thus preventing culture identification. These methods are also time consuming and costly.

Both types of commonly used light microscopy procedures involve the coupling of specimens with appropriate stains. Bright field light microscopy can detect mucoid-slime production by bacteria when stained with ruthenium red, toluidine blue, crystal violet or other compounds which are sensitive to an acid mucopolysaccharide composition, while other stains (Gram, periodic acid Schiff, hematoxylin and eosin) are specific for the cells enmeshed within biofilms (56,148,151). The use of epifluorescent light microscopy has been described in new rapid methods for detecting and quantifying biofilm bacteria on catheter surfaces (129). The fluorescent agents (calcifluor white, acridine orange) stain the background surface and are then coupled with a counterstain (Evans blue, malachite green) for specific cell identification (129,148).

Radioisotope labelling of bacterial substrates ($^{3}H/^{14}C$-amino acids and sugars) for the evaluation of metabolic activity can be used to enumerate and assess the function of adherent bacterial populations (7,130,166). However, this process also has drawbacks as it requires the use of radioactive materials which are hazardous and expensive, and that the microorganism in question be able to transport and metabolize the substrate which has been

labelled. Furthermore it is unknown whether the presence of labelled substrates will alter cellular function.

Spectrophotometry has been used by Christensen et al and others to quantitate bacterial adherence and measure the variations of mucoid-slime production amongst bacterial strains (31,35,153). Maki et al and others developed the semiquantitative technique of streaking medical implants which are suspected of harbouring biofilm bacteria onto solid agar media and expressing the results of cultures as the number of adherent bacteria per surface area of specimen (73,137,199). However, it cannot be assured that this technique would dislodge a representative population of the adherent biofilm microorganisms nor provide an accurate numerical assessment (185). The flushing of suspected biofilm-laden implants is yet another process which cannot guarantee the complete dislodgement of microorganisms, thus potentially providing false-negative results (39).

Tollefson et al and others have used low energy, high frequency ultrasonic oscillation to cause dislodgement of surface adherent microbes (194,211). Using the Robbins device, Holmes et al developed biofilms on silicone disks in vitro (109). They homogenized these disks by blender to separate bacteria from the exopolysaccharide slime. Still others have caused mechanical disruption of biofilms by scraping the surfaces of implant devices and subsequently culturing the recovered material (129). However, these methods would not always concur with SEM observations and vice versa, thus indicating discrepancies in sensitivity

between the various techniques (51,94).

In general, all the aforementioned procedures for studying bacterial biofilms fall short in one way or another to meet the requirements of an optimal method of investigation: simplicity; accuracy; economy; in situ assessment; determination of viability; and potential applicability as a routine microbiological technique.

1.3.2 Tetrazolium Derivatives as Indicators of Metabolism.

The method described here for the study of bacterial biofilms in their intact state involves the use of a colourless cyclic compound which couples with bacterial metabolic processes resulting in the formation of a deeply pigmented precipitate which can be observed under suitable conditions. This precipitate is indicative of active metabolic function and interpreted as bacterial viability. A standard microbiological agar medium is coupled with the compound thus formulating the basic method used to study biofilm bacteria. The chemical compound which was used in this new method is 2,3,5 triphenyltetrazolium chloride (TTC), one of a large class of tetrazolium salts. This is a novel application of this tetrazolium derivative to demonstrate bacterial oxidative metabolism in a glycoprotein matrix.

Tetrazolium salts are quaternized tetrazoles with a five membered ring, consisting of one carbon and four nitrogen atoms as the basic structure (158). One of the four nitrogens in the ring is quaternary, and thus provides for salt-like properties (135). Tetrazolium salts are water soluble, yielding a colorless aqueous solution and as such are not dyes in themselves (158). When reduced however, these salts form deeply pigmented end products known as formazans (167). Formazans are water insoluble compounds containing the characteristic molecular sequence -N=N-C=N-NH

(158,167). The basic chromophore in this chain is the azo-group (-N=N-) (135).

Tetrazolium salts, including TTC, were first prepared by Pechman and Runge in 1894 (quoted in 125,126). TTC has a molecular weight of 334.81, is lipid soluble (thus penetrating cell membranes), and will undergo photoreduction when exposed to visible light (8,158,167). Discovering that tetrazolium salts would undergo characteristic reduction to formazans in biological reactions, led to the applications of TTC in most of the early experimental botanical research of the 1940's (125,126).

Tetrazolium salts were first used to demonstrate the viability of seeds (48). Exposure of germinating seeds to aqueous TTC resulted in colouring of the viable tissues to deep red (158). In 1944 the redox potential for typical tetrazolium salts was measured with hydrogen electrodes to be -0.08 volts (112). This value corresponded to those of biological processes in living cells and indicated that TTC was capable of acting as an artificial electron receptor for the pyridine nucleotide linked enzyme systems (106,146,158).

The process of cellular respiration involves the transfer of electrons from organic substrates to electron acceptor compounds for the generation of energy. In mammalian cells the final electron acceptor is solely oxygen, whereas for bacteria the final electron acceptor is species dependent and may include oxygen, other organic compounds or inorganic substances. The changes in reduction-oxidation (redox) potential yields electrons

necessary for the phosphorylation of adenosine diphosphate to adenosine triphosphate, the basic energy unit of all cells. There are several types of electron carriers, including nicotinamide adenine dinucleotide (NAD), active in various dehydrogenases; flavin mononucleotide (FMN) in NADH dehydrogenases; ubiquinone; and finally the successive cytochrome complexes (15,131).

Several key experiments supported the involvement of TTC with cellular enzymatic processes (22,23,66,67,68,85,127,128,195). Mammalian and plant tissues which were heated above 82°C lost their ability to reduce TTC, thus demonstrating a causal relationship between the known destruction of enzymatic activity with high temperatures (146,158). There was differential reduction of TTC by carcinomatous tissue as compared to the rate exhibited by surrounding healthy tissues, indicating a variation of metabolism (18,98,158,202). The infusion of TTC into blood will only stain leukocytes and not plasma or red blood cells, confirming the need of oxidative metabolism for the staining reaction to occur (98,158). TTC reduction would proceed in the vicinity of still living or very recently dead cells after the cessation of circulation (202). Researchers theorized that TTC interferes with the cell's normal oxidative metabolism and hence a decreased oxygen supply would provide less competition for TTC reduction (127). Indeed, TTC was later shown to be reduced more efficiently under anaerobic conditions, becoming the sole competitor for cytochrome oxidase (22).

Throughout the late 1940's and 1950's many investigators showed by the homogenization of various plant and animal tissues that various enzymes were associated with TTC reduction (223). Tetrazolium salts were used as a simple and rapid method of measuring the enzymatic activity through colorimetry and assessing the histochemical localization of enzymes such as monoamine oxidase, succinic dehydrogenase and lactic dehydrogenase amongst others (22,23,85,106,128,135,195,227). However, in 1956 elegant and detailed work by Farber et al demonstrated that the transfer of electrons to and subsequent reduction of TTC was actually due to enzymes responsible for reducing the electron carrier complexes and did not directly involve dehydrogenases or the electron carriers (NAD, FMN, cytochromes) themselves (29,66,67,68,84). Confirmation of the location of TTC reduction was shown in several experiments using various metabolic inhibitors to block the flow of electrons at different regions along the respiratory enzyme chain (29). Thus TTC would not only be classified as a vital stain, but more specifically as an indicator of active enzymatic processes.

Since the value of a rapid indicator to assess cellular viability of vegetation was recognized in the 1940's, the applications of TTC have since multiplied (181). By 1948 the chemical had become especially valuable in bacteriological research. In an actively growing bacterial culture the reducing activity is proportional to the number of microorganisms in the culture, assuming that a uniform

metabolic state exists (208). The number of microorganisms in a bacterial culture also demonstrates a positive correlation with the amount of tetrazolium salt which is reduced, and thus the degree of color intensity developed (123,167). A characteristic which differentiates the rapidly multiplying cells in a bacterial culture from the older cells is the higher rate of oxygen uptake and thus metabolism in the former. This property of bacterial cultures was explored with TTC, recording the degree of formazan precipitate as variations in cell viability (123). This application of TTC demonstrated that the older culture of mycelium of Penicillium chrysogenum yielded the most penicillin (74).

In the early 1950's TTC was being added to motility media to aid in the detection of motile bacteria (119,135). As bacteria grew on the medium, the tetrazolium salt was incorporated into the cells and reduced to the insoluble red pigmented formazan. The red precipitate was formed only in those areas of the media in which bacteria were growing. Tetrazolium salts were then incorporated into other bacteriological culture media to facilitate studies of colony variation as well as to expedite detection and enumeration (167). It is significant to note that colonies coloured by the presence of formazan precipitates remained viable on subculture (226).

For studies of cellular metabolism, TTC was applied to indicate bacterial inhibition in the presence of antimicrobials through a deficiency in reducing potential

(183). This could be quantitatively measured with colorimetry by the difference in reduction as compared to healthy, non-exposed cultures. Additionally, standard penicillin assay plates which were incubated and then flooded with an aqueous TTC solution promptly outlined the zones of inhibition by a bright red ring, while the inhibition zones themselves remained uncoloured (181). Similar culture plates which were first treated with formaldehyde solutions to inactivate enzyme systems showed no colour changes following exposure to aqueous TTC (181). In 1973 the Kirby-Bauer susceptibility test was similarly modified by the addition of tetrazolium salts to the surface of culture mediums following antimicrobial exposure. The major advantage gained over the current method was a more rapid determination of bacterial susceptibility, thus decreasing the culture time required before an optimal antimicrobial could be chosen for therapy (10,11,20,83). TTC has also been used for simple and rapid tests of bacteriuria; for the detection of bacteria in milk and foods; to determine antibacterial activity of disinfectants; and to accelerate the diagnosis of tuberculosis (167).

Technically, TTC is not found to be suited for specific intracellular localization of individual reducing centers because it is precipitated in aggregates within the potential space between the cell wall and the cytoplasmic membrane (224). However, TTC can be a most efficient chemical for the differentiation of viable and non-viable microorganisms (Appendix 3.A.4).

In general, redox indicators such as TTC inhibit growth because of their ability to sidetrack the normal mechanisms of electron transfer (189). Investigations (103,147) of the influence of several tetrazolium compounds on the growth of various microorganisms demonstrated that monotetrazolium salts, such as TTC, were the least toxic and did not adversely affect staphylococci at concentrations which were ten times those used to assess bacterial biofilm viability, as described in this thesis.

CHAPTER 2

AIMS OF THESIS

In the past decade continuous ambulatory peritoneal dialysis has become an accepted alternate form of renal replacement therapy for patients with end-stage renal disease. Despite recent advances in materials and techniques, peritonitis has remained the major complication of this treatment modality. Coagulase-negative staphylococci, principally S. epidermidis, are the most frequent pathogens associated with CAPD peritonitis. They are also increasingly prevalent in foreign body infections, often residing on the implanted material (including CAPD catheters) in a biological film. Although their relative contribution remains unclear, bacterial biofilms have been implicated as a possible source of persistent and recurring infection.

The main focus of this thesis was to study the effects of agents administered to CAPD patients on S. epidermidis biofilm preparations using a newly developed method. The aims of the succeeding sections in the thesis are represented by the following:

1. To develop a new method for the formation and investigation of bacterial biofilms in vitro which would obviate the requirements for specialized equipment and materials. This method would have

general microbiological applicability and thus accomodate large numbers of variables concurrently.

2. To assess the efficacy of several classes of antimicrobials against S. epidermidis existing in two different phases of growth: fluid versus biofilm.
3. To extensively evaluate any antimicrobial agent demonstrating superior activity against S. epidermidis biofilms. These investigations would include kinetic studies, variations of exposure media and the potential for synergy.
4. To develop a mouse model of infection for investigation of intraperitoneally implanted catheter adherent S. epidermidis biofilms. This would entail an assessment of host response to biofilm bacteria and the evaluation of currently recommended therapeutic regimes in persisting or recurrent S. epidermidis peritonitis. In addition, the in vivo assessment would include drugs of high antimicrobial activity against S. epidermidis biofilms as determined in the initial in vitro studies.
5. To investigate the cytotoxicity of fresh peritoneal dialysis solutions and common but variously administered non-antibiotic i.p. drug additives in a new method involving mouse peritoneal cells.

CHAPTER 3

MATERIALS AND METHODS

3.1 Bacterial Biofilm Assay

3.1.1 Bacteria:

A clinical isolate of Staphylococcus epidermidis recovered from the peritoneal dialysis effluent of a patient with CAPD peritonitis was used throughout these experiments. Identification was confirmed with standard laboratory techniques including Gram stain, catalase and coagulase reaction, morphology, differential growth and the Vitek test system. Further characterization of this isolate is presented in Appendix 1. Upon subculture this strain was shown to be a prototypic producer of slime and fully sensitive in the fluid phase to all appropriate antibiotics by routine sensitivity testing. To discount strain variation identical aliquots of this coagulase-negative staphylococci (C-NS) were frozen and stored at -70°C in 10% glycerol broth. Cultures were propagated from thawed aliquots onto 5% defibrinated horse blood agar and subcultured twice prior to experimentation to ensure purity.

3.1.2 Preparation of Standardized S. epidermidis Biofilms:

Standardized bacterial biofilms were formed on alcohol sterilized soda glass microscope slides (Fisher #12-552) individually contained within Petri dishes. Working

aseptically each dish was half filled with 20 ml of glucose enriched growth medium, tryptone soya broth (TSB, CM129, Oxoid Ltd., England). From an overnight suspension of 10^8 CFU S. epidermidis in peptone water, an aliquot of 10^4 CFU in the log phase of growth was seeded into the individual dishes and incubated for 18 hours at 37°C. It was determined that this method would yield reproducible uniform and confluent S. epidermidis biofilms of minimal density.

The characteristic pattern of growth of this particular strain of S. epidermidis from the seeded TSB to the slide surface was identified by Gram and toluidine blue staining. Sequential hourly cultures indicated the first adherent bacterial cells after 3 hours of incubation. By 6 hours the adherent population of bacteria per slide increased but without the presence of microcolonies; these appeared after 9 hours of incubation. Twelve hours of growth showed a confluent population of adherent cells with micro- and macrocolonies in close proximity. The detection of an amorphous extracellular slime-like substance by toluidine blue staining was first noted at 14 hours of incubation. This substance completely coated the slide by 18 hours, encasing the adherent colonies of S. epidermidis and as such defined "minimal density" biofilm formation (Figure 1).

Biofilms were only formed on the upper surface of the horizontally incubated slides. With minimal manipulation the individual preparations could be exposed to various combinations of antimicrobials and test milieus at variable

concentrations. Each dish could then be incubated at different temperatures for set periods of time. For comparative purposes and to demonstrate the general applicability of this method, S. epidermidis biofilms were also formed on custom-made Silastic-coated glass slides. This Silastic compound is the basic material of Oreopoulos-Zellerman CAPD catheters (Accurate Surgical Instruments Corp., Toronto). In selected in vitro and in vivo experiments S. epidermidis biofilms were developed on uniform 12 mm long segments of Oreopoulos-Zellerman catheters, bevelled at the ends to remove rough surfaces (Accurate Surgical Instruments Corp., Toronto) (Figure 2A). These experiments demonstrated the versatility of this model for forming biofilms on surfaces of different shapes and chemistry.

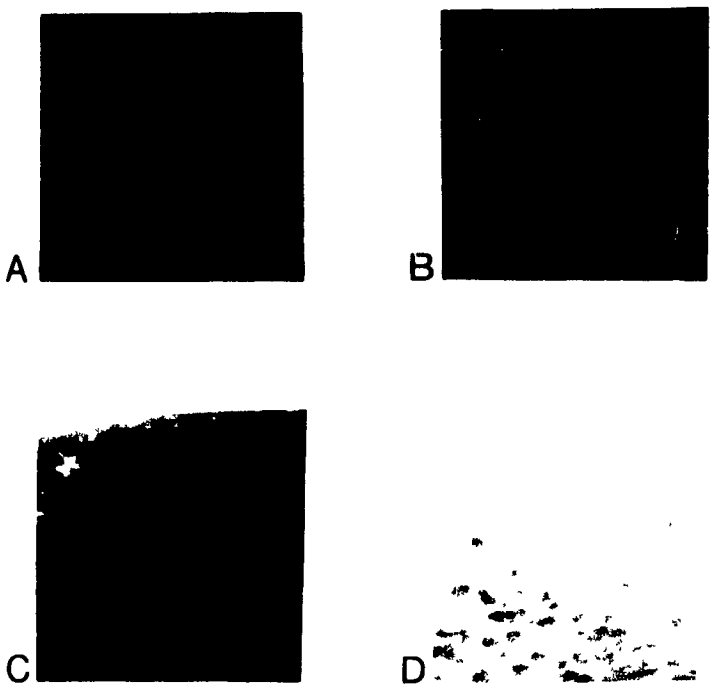

Figure 1. The in vitro development of S. epidermidis biofilms on plane glass surfaces is indicated by toluidine blue staining. Panels A and C represent 12 hours of bacterial growth, showing the relative size of adherent macrocolonies and the absence of exopolysaccharide matrix, respectively. Panels B and D are representative of "minimal density" bacterial biofilms after 18 hours of growth. The relative opacity of panel D in comparison to the transparency of panel C is an indication of exopolysaccharide production by the adherent colonies. The increased size and number of developing colonies between 12 and 18 hours growth is contrasted in panels A and B.

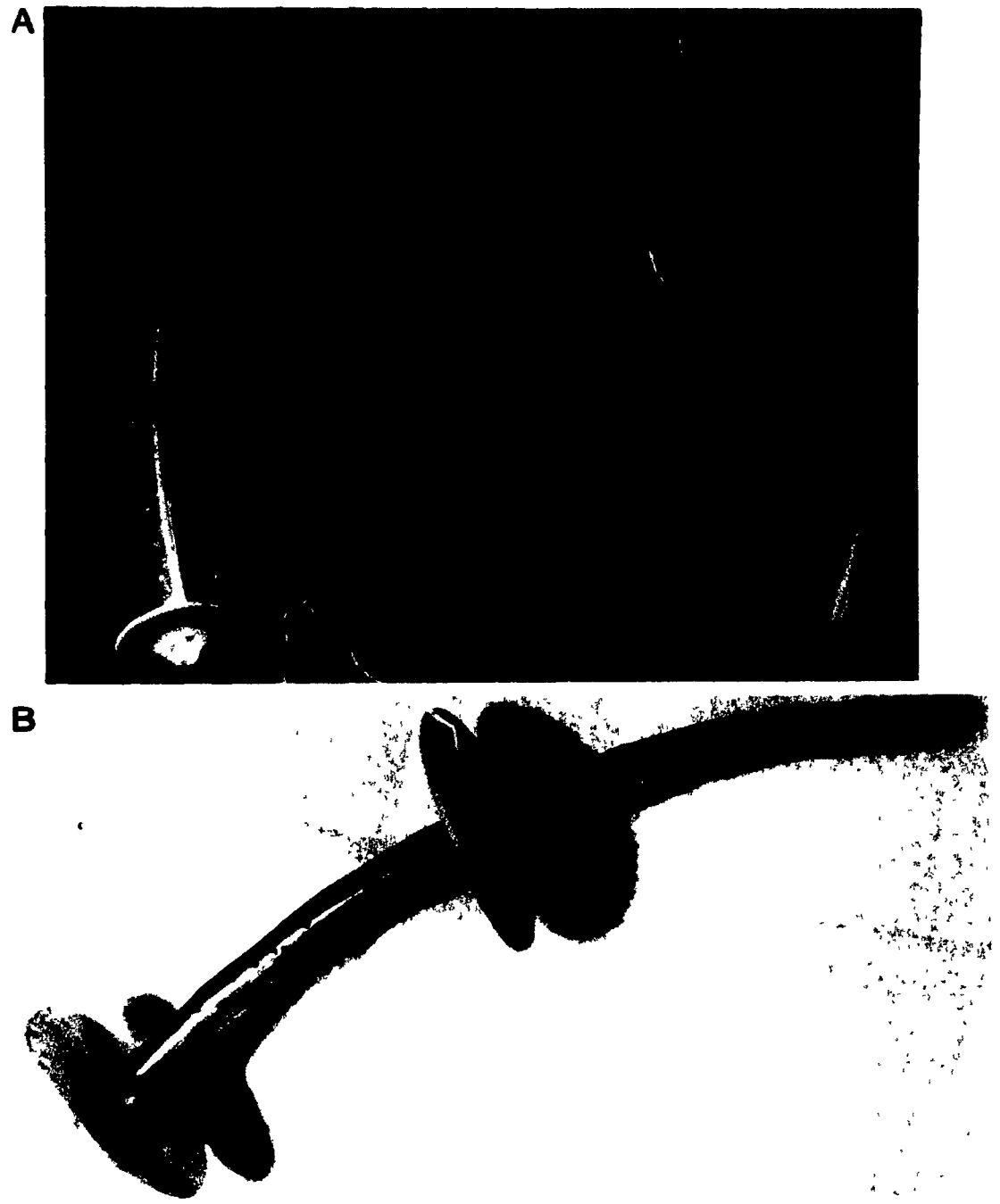

Figure 2. A, Oreopoulos-Zellerman CAPD catheter and custom-made 12 mm segment used for *in vivo* animal studies. B, Intraabdominal segment of an Oreopoulos-Zellerman CAPD catheter removed from a patient with peritonitis. Incubation with TTC broth demonstrated an adherent biofilm on the luminal and extraluminal surfaces. Subsequent subcultures indicated the pathogen as a *Candida albicans* species.

3.1.3 Antimicrobials and Test Media:

Following 18 hours of incubation in the growth media (TSB), the standardized S. epidermidis biofilms were vigorously rinsed twice in sterile distilled water to remove excessive loosely-attached surface slime and bacteria, and allowed to drain of fluid but not dry. Under sterile conditions these biofilms were immersed into antimicrobial solutions at various concentrations and allowed to incubate for periods of time ranging from 5 seconds to 24 hours at different temperatures.

Two main classes of antimicrobials, antibiotics and antiseptics/disinfectants including a recently developed series of compounds (RenNew-P), were examined for their activity against S. epidermidis biofilms.

Antibiotic solutions were prepared from commercially available sensitivity discs and reference material of known potency, diluted in the standard control medium, peptone water (1% protein digest in normal saline, CM9, Oxoid Ltd., England). Thirty five antibiotics were assessed for their antimicrobial activity against standardized S. epidermidis biofilm preparations. The concentrations used ranged from recognized MIC values to being equal to or higher than tissue concentrations expected in clinical practice. The concentrations (μg/ml) are expressed in parentheses following the name of the antibiotics and are representative of the upper limit used: amikacin (60), ampicillin (30), bacitracin (20), cefadroxil (30), cefamandole (60), cefazolin (30), cefoperazone (30), cefotaxime (30),

cefsulodin (30), ceftazidime (30), cefuroxime (30), cephalexin (30), chloramphenicol (20), ciprofloxacin (12.5), clindamycin (20), cloxacillin (20), erythromycin (45), fusidic acid (30), gentamicin (30), imipenem (20), moxalactam (30), neomycin (30), norfloxacin (20), novobiocin (60), oleandomycin (45), oxacillin (20), penicillin G (25), rifampin (20), spectinomycin (20) streptomycin (20), sulbactam with ampicillin (20, 20), tetracycline (60), tobramycin (20), trimethoprim-sulfamethoxazole (5, 25), and vancomycin (60).

For the detailed examination of several antibiotics, alone and in synergy studies, solutions were prepared from intravenous formulations standardized by reference assay (clindamycin: Upjohn; cloxacillin: Beecham; gentamicin: Schering Canada Inc.; rifampin: Ciba-Geigy, Merrell-Dow, Sigma; rifamycin SV: Sigma; tetracycline: Lederle; and vancomycin: Eli Lilly & Co.) and developmental analog formulations (rifapentine: Merrell Dow; CGP029861: Ciba-Geigy).

The rifamycin family of antibiotics possess an aromatic double ring system, spanned by a long aliphatic bridge or loop called the ansa ring plus a short side-chain permitting substitution at two sites. Substitutions at either or both of these two sites provides for the variations in semisynthetic derivatives of the parent rifamycin (120,186,187,196). These derivatives include rifampin, rifapentine and other experimental analogs (CGP029861) (Figure 3).

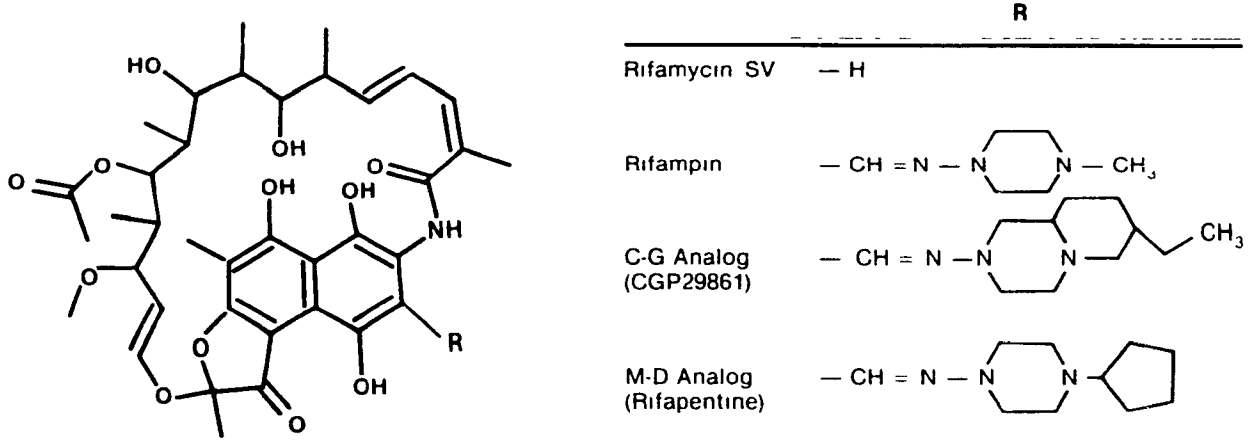

Figure 3. Chemical structure of rifamycin antibiotics.

Commercially available antiseptics and disinfectants were tested at normal working strength for their antimicrobial activity against similar biofilm preparations. The list of agents investigated include: Amuchina 50%, chlorhexidine 0.05%, Dakin's solution 0.5%, formol saline 4%, hydrogen peroxide 3%, and povidone-iodine 10%.

A new series of antiseptic/disinfectant products were also evaluated. They consisted of four formulations of RenNew-P, which constitute variations of chlorous acid/chlorine dioxide generating systems (Alcide Corp., Norwalk, Conn., USA). The compounds were investigated both undiluted and diluted with our standard control media.

The efficacy of antibiotics and the RenNew-P formulations was further evaluated in various other testing milieus for their activity against S. epidermidis biofilms. These milieus included fresh and spent peritoneal dialysis (PD) solutions. The fresh PD solution (Inpersol, Abbott Laboratories, Canada) contained lactate as the buffering agent and various dextrose concentrations (0.5, 1.5, 2.5 and 4.25%). Spent PD fluid consisted of the effluent recovered following the intraperitoneal (i.p.) dwelling of PD solution. Spent PD fluid was obtained from uninfected patients in the Dialysis Unit of the Montreal General Hospital. The fluid was screened to ensure the absence of possible antimicrobials, centrifuged at 300g x 10 min at 4°C to remove fibrin clots and cells. The processed fluid of 12 patients was subsequently pooled and stored in aliquots at -20°C. The average duration of the i.p. dwell period was 8

hours.

Fresh PD solution was also modified by the addition of serum and buffer to further explore the possible effect of these constituents of spent PD fluid on antimicrobial activity. Fresh PD solutions were supplemented with heat inactivated foetal calf serum (Flow) at concentrations which are representative of the protein content in peritoneal dialysis effluent of uninfected patients (0.05%) and the effluent recovered during episodes of peritonitis (0.25%). The acidic property of fresh PD solution was also adjusted by titration with 20 mM HEPES buffer (Flow) to a neutral pH. In addition to the aforementioned milieus, the activity of RenNew-P formulations was also compared in 0.9% normal saline.

3.1.4 Drug Additives to PD solutions:

The following non-antibiotic drugs are currently, but variously administered i.p. for the management of CAPD patients. These PD drug additives were examined in the aforementioned fresh and spent PD solutions individually and in combination with rifampin, for their efficacy against S. epidermidis biofilm preparations. They include: calcitriol (investigational drug, Abbott Laboratories) (54), desferrioxamine mesylate (Desferal, Ciba-Geigy) (16,105,204,205), furosemide (Lasix, Sabex) (97), heparin sodium (Organon Canada Ltd.) (76), insulin (Insulin-Toronto, Novo Laboratories Ltd.) (4,57,71,104), phosphatidylcholine (kindly supplied by Dr. A. Cantaluppi, Rome, Italy) (58),

streptokinase (Streptase, Hoeschst Canada Ltd.) (162,225), urokinase (Abbott Laboratories) (175), verapamil hydrochloride (Isoptin, Knoll Pharmaceuticals Canada Ltd.) (165). The concentrations chosen for test purposes incorporated the range of dosages which are clinically administered.

3.1.5 Assessment of Antimicrobial Activity:

Following the designated exposure times, individual biofilms were removed from test solution (again under sterile conditions), rinsed twice in sterile distilled water and allowed to drain of fluid but not dry. With sterile forceps the biofilm coated surface of the slide was placed horizontally onto a flat recovery-indicator medium (R-IM) and returned for incubation at 37°C for a period of 24 hours.

The R-IM consists of a nutrient agar base (Difco) which has been supplemented with 2,3,5 triphenyltetrazolium chloride (TTC, Mallinckrott Inc., Kentucky, USA). The agar base functions to provide essential bacterial substrates as well as allowing for the dispersal of any residual antimicrobials which may remain associated with the biofilm matrix following the rinsing procedure. The historical development, physicochemical properties and applications of TTC have been extensively reviewed in Chapter 1.3.2. In brief, the chemical is only incorporated by metabolically active microorganisms in which it undergoes reduction to form a coloured precipitate and as such is an indicator of

bacterial viability (Figure 4).

The R-IMs were prepared within Petri dishes. 0.5 ml of sterile 1% aqueous solution of TTC was added aseptically for every 100 ml of nutrient agar following sterilization of the medium (to prevent the possible reduction of the chemical). For special studies the R-IM, usually at a pH of 7, was titrated down to pH 6 and 5 by the addition of 0.1N HCl.

Three patterns of response were observed for the exposed biofilms following incubation on the R-IM: no metabolic activity at 24 hours was indicative of complete biofilm killing; metabolic activity at 4 hours was considered to represent fully viable biofilms; and the absence of metabolic activity at 4 hours but full recovery at 24 hours was interpreted as metabolic inhibition. Positive and negative control biofilms were included in all assays, and consisted of biofilms exposed to 1% peptone water and 4% formol-peptone for 24 hours, respectively (Figure 5).

The basic R-IM technique was further modified by incorporating TTC into liquid culture media for the determination of viable microorganisms on the surface of catheter segments recovered from animals (Chapter 4.2) and from CAPD patients (Figure 2B).

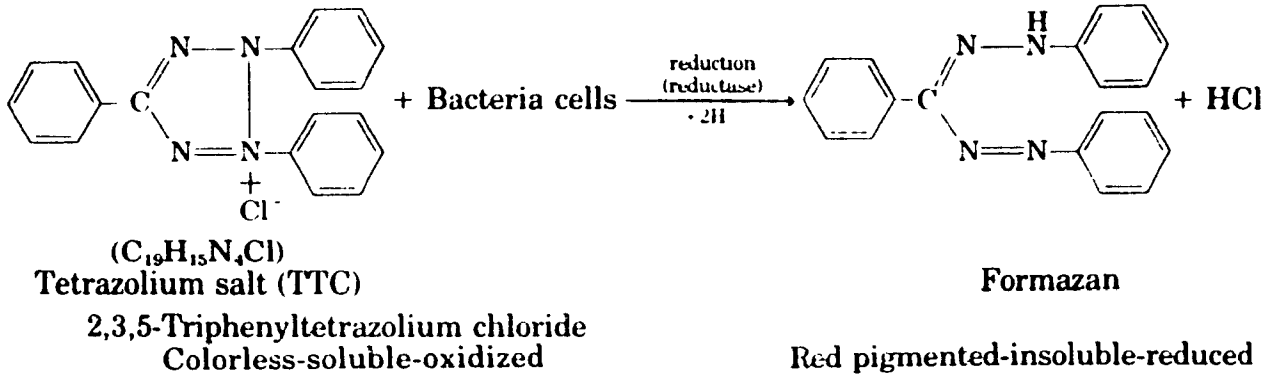

Figure 4. The chemical reduction of 2,3,5 triphenyltetrazolium chloride to formazan.

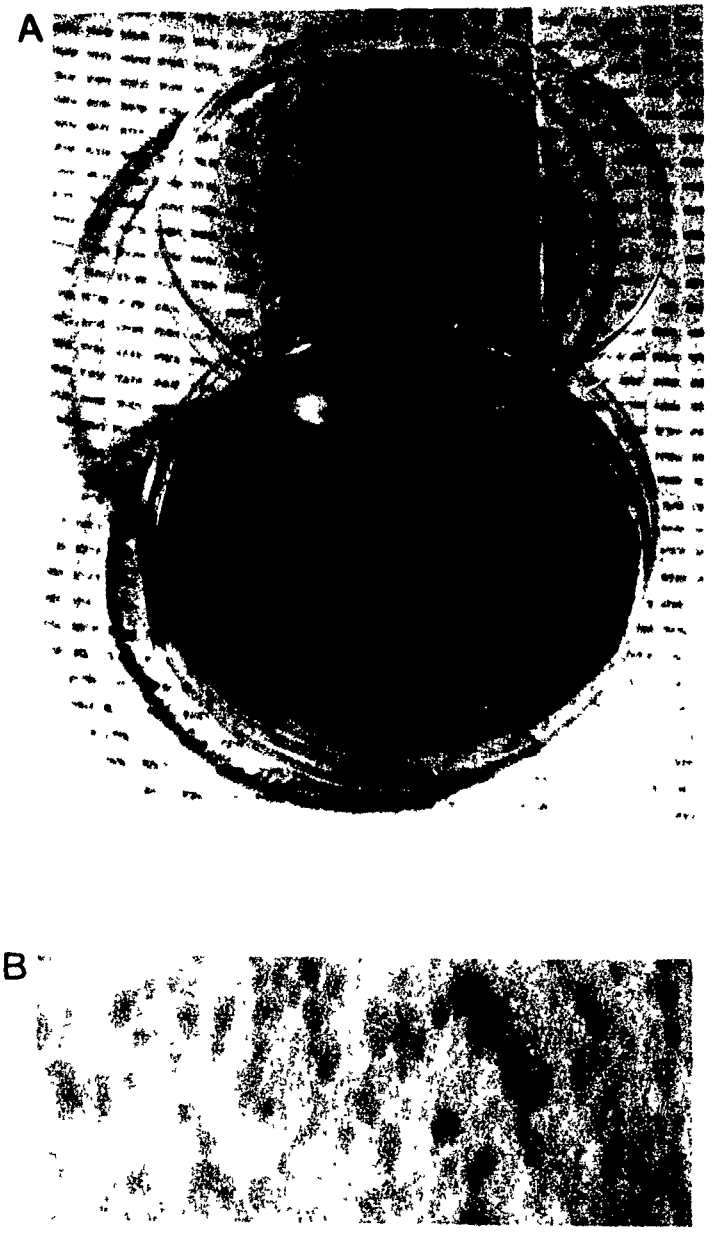

Figure 5. A, Petri dish containing the R-IM and on its surface a fully viable S. epidermidis biofilm which has incorporated the colourless TTC salt and formed the insoluble red formazan precipitate within an hour of incubation.
B, Magnification (x 10) of the biofilm slide shown in panel A, demonstrating various sizes and shapes of colony development. The exopolysaccharide matrix is not stained by TTC. The yellow background is due to the nutrient agar component of the R-IM.

3.2 *In vivo* Evaluation of *Staphylococcus epidermidis* Biofilms in the Mouse

Five week old female C57BL/6 inbred mice were obtained from Charles River Breeding Laboratories (St-Constant, Québec) and housed in groups of 6 within standard hanging cages. The animals were acclimatized for an additional week while permitted free access to tap water and mouse chow.

To obtain standardized *S. epidermidis* biofilm preparations in the animals, bacterial biofilms were grown *in vitro*, as previously described, onto custom-made Silastic catheter segments before implantation into the peritoneal cavity. The catheter segments were grouped according to 1, 2 and 3 day biofilms, a 5 second immersion in a suspension of 10^8 CFU *S. epidermidis* and sterile controls.

The peritoneal implantation of catheter segments was performed with mice under light ether anesthesia. A cardinal feature of this procedure was the placement of the segments entirely within the peritoneal cavity without exit at the skin. Using a left flank approach, the upper end of each segment was anchored to the posterior abdominal wall with a sirgle suture passing through a side perforation in the catheter. Care was taken to minimize damage to the bacterial biofilms and avoid contact with the surgical wound. The deep layers of the abdominal wall were closed with a running suture and clips were applied to the skin.

Antibiotherapy was not instituted until 7 days after catheter implantation.

Injectable forms of rifampin (Ciba-Geigy, Toronto) and vancomycin hydrochloride (Eli Lilly & Co.) were prepared in 0.9% sodium chloride. The antibiotics were administered i.p. through the anterior abdominal wall directly into the lumen of the catheter segment which was easily palpated through the intact skin. A dose of 15 mg/kg/day was given at 12 noon for a period of 7 days. The total volume was adjusted to 1.0 ml per dose. Only animals constituting the group with sterile implanted catheter segments and the group with 3 day S. epidermidis biofilm/catheter-complexes underwent therapy.

Sacrifice was performed by cardiac exsanguination with mice under ether anesthesia. Routine hematology was performed with a Coulter counter (Model 2B1 Coulter Electronics Inc., Hialeah, Fla., USA). Peritoneal washings with modified minimal essential medium (Flow) were processed immediately for bacteriology and cell enumeration. Samples were plated in duplicate onto blood agar and incubated at 37°C for evaluation for superficial viable bacterial counts. Numerical assessment of the peritoneal cell populations was performed in Neubauer counting chambers and differential cell counts were determined from cytocentrifuge preparations stained with Diff-Quick (American Scientific Products).

The catheter segment was carefully removed following fine dissection of adhering tissue and sectioning of its anchoring suture to the abdominal wall. The catheter

segment was then assessed for the presence of metabolically active S. epidermidis biofilms. The segment was first vigorously rinsed in TTC-containing culture broth to dislodge loosely associated microorganisms from the surface and thereafter placed into a similar but separate TTC-containing culture broth for detection of surface adherent microorganisms. Both the rinse medium and the medium containing the catheter segment were incubated at 37°C for up to 5 days.

3.3 Cytotoxicity and Functional Assays of Mouse Peritoneal Cells

3.3.1 Animals:

C57BL/6 female inbred mice were obtained from Charles River Breeding Laboratories (St-Constant, Québec) at 35 days of age. The mice were left to acclimatize within holding facilities for a period of one week prior to experimentation, and were permitted free access to tap water and mouse chow.

3.3.2 Surgical Induction of Renal Failure:

Renal failure was induced by two separate surgical procedures as previously described (77,78). In brief, the right kidney was approached through a right flank incision while the mice were under ether anesthesia. The kidney was separated from surrounding structures with fine forceps dissection with special care not to damage the adrenal gland or the ureter. A single point cauterizer (Hyfrecator, Model X-712, Birtcher Corp., Los Angeles, Calif., USA) was applied systematically to the entire kidney surface. The deep planes of the abdominal wall were closed with a running suture, sprayed with Neosporin (Burroughs Wellcome Inc.) and the skin was closed with surgical clips. A recovery period of 2 weeks was allowed prior to the second surgical procedure, during which time the animals were maintained on the aforementioned diet. For the second surgery the animals

were randomized to either renal failure or sham surgery groups. A left nephrectomy was performed on the animals of the renal failure group and simple visualization of the left kidney in the sham-operated mice. Six to 10 weeks were allotted after the second surgery before the animals were used for experimentation.

3.3.3 Test Media and Drug Additives to PD solutions:

The testing media consisted of either fresh PD solution (Inpersol, Abbott Laboratories) which incorporated a range of dextrose concentrations (0.5-4.25%) or phosphate buffered saline (PBS, 1 x Dulbecco's Formula, pH 7.4, Flow). These solutions were evaluated independently and in separate combinations with the nine non-antibiotic therapeutic drugs variously added to PD solutions as mentioned previously in Chapter 3.1.4.

3.3.4 Peritoneal Cell Populations:

The harvesting of resident and elicited peritoneal cell populations was performed by the standard technique of peritoneal lavage. This procedure involved the instillation of 10 ml cold PBS through the suprapubic fat pad with a 20 gauge needle. Following one minute of equilibration the fluid was aspirated. To obtain an elicited population of peritoneal cells with a majority of PMN leukocytes (cytotoxicity assay), animals received an i.p. injection of 1 ml volume of 3% Brewer's thioglycolate broth (Difco) 18 hours prior to experimentation. To obtain a majority of

elicited macrophages (functional assay) the peritoneal lavages were performed 72 hours after injection. The magnitude of the inflammatory response in vivo was determined by counting the total number of cells recovered from the peritoneal lavage using a hemocytometer. Differential counts were performed on cytospin preparations using a Diff-Quick (Harleco) staining procedure.

3.3.5 Cytotoxicity Assay Procedure:

For each assay the peritoneal lavages of 10 mice were pooled. Following enumeration, the pooled cells were centrifuged at 300g x 10 min at 4°C, and then resuspended at a concentration of 2×10^7 cells/ml in cold PBS. 50 µl aliquots of the cell suspension were added to 500 µl of the test solutions in 5 ml polypropylene tubes at 37°C. Each test situation involved a zero-time control and a 60 minute incubation at 37°C. At the respective endpoints the reaction was arrested by the addition of 3 ml cold minimal essential medium, Eagle (modified), with Earle's salts and glutamine (MEM, Flow) containing 10% heat inactivated foetal calf serum (FCS, Flow) and buffered with 20 mM HEPES (Flow). In each instance the tubes were immediately centrifuged (300g, 4°C, 10 min), the supernatants discarded and the pelleted cells resuspended in 50 µl of cold medium. Cell viability was determined by the standard trypan blue exclusion method.

3.3.6 Functional Assay Procedure:

The production of hydrogen peroxide (H_2O_2) was determined on adherent macrophages using phorbol myristate acetate as a stimulant, 60 minutes after the exposure to the various test media and drug additives to PD solutions described in Chapter 3.3.3. H_2O_2 production was measured using already well described spectrophotometric techniques (149,174). Results are expressed as nmoles/mg protein. The amount of protein was determined by the Bradford technique (21) after lysing the macrophage monolayer with 1 ml of 0.5N NaOH.

CHAPTER 4

RESULTS

4.1 *In vitro* Assessment of Antimicrobial Activity Against *Staphylococcus epidermidis*: Fluid Versus Biofilm Phases

4.1.1 Activity of antibiotic screen against *S. epidermidis*.

In marked contrast to antibiotic sensitivity testing with *S. epidermidis* in the fluid phase, we found that 34 of the 35 antibiotics tested showed no activity against this bacteria in the biofilm phase, even after 24 hours of exposure. Rifampin was a striking exception demonstrating pronounced antimicrobial activity. The range of antibiotics tested is presented in Table 1. The concentrations used were equal to or higher than that which may be obtainable in tissues during clinical practice. The naked eye and light microscopy appearances of selected control and antibiotic-exposed *S. epidermidis* biofilms are contrasted in Figure 6.

Table 1. Antimicrobial activity of 35 antibiotics during 24 hours exposure against S. epidermidis: fluid phase vs biofilm.

Antibiotics*	Concentrations (µg/ml)	Fluid phase**	Biofilm***
amikacin	60	-	+
ampicillin	30	-	+
bacitracin	20	-	+
cefadroxil	30	-	+
cefamandole	60	-	+
cefazolin	30	-	+
cefoperazone	30	-	+
cefotaxime	30	-	+
cefsulodin	30	-	+
ceftazidime	30	-	+
cefuroxime	30	-	+
cephalexin	30	-	+
chloramphenicol	20	-	+
ciprofloxacin	12.5	-	+
clindamycin	20	-	+
cloxacillin	20	-	+
erythromycin	45	-	+
fusidic acid	30	-	+
gentamicin	30	-	+
imipenem	20	-	+
moxalactam	30	-	+
neomycin	30	-	+
norfloxacin	20	-	+
novobiocin	60	-	+
oleandomycin	45	-	+
oxacillin	20	-	+
penicillin G	25	-	+
rifampin	20	-	(-)
spectinomycin	20	-	+
streptomycin	20	-	+
sulbactam with ampicillin	20, 20	-	+
tetracycline	60	-	+
tobramycin	20	-	+
trimethoprim-sulfamethoxazole	5, 25	-	+
vancomycin	60	-	+
controls: 4% formol saline		-	-
1% peptone water		+	+

*All antibiotics were eluted from commercially available sensitivity discs in 1% peptone water.
**10^8 CFU inoculum of S. epidermidis.
***Standardized 18 hour S. epidermidis biofilm preparations.
Results are expressed as:
+ : viable, full metabolic activity.
(-): predominantly non viable, minor foci of resistance.
- : non viable, bactericidal effect.

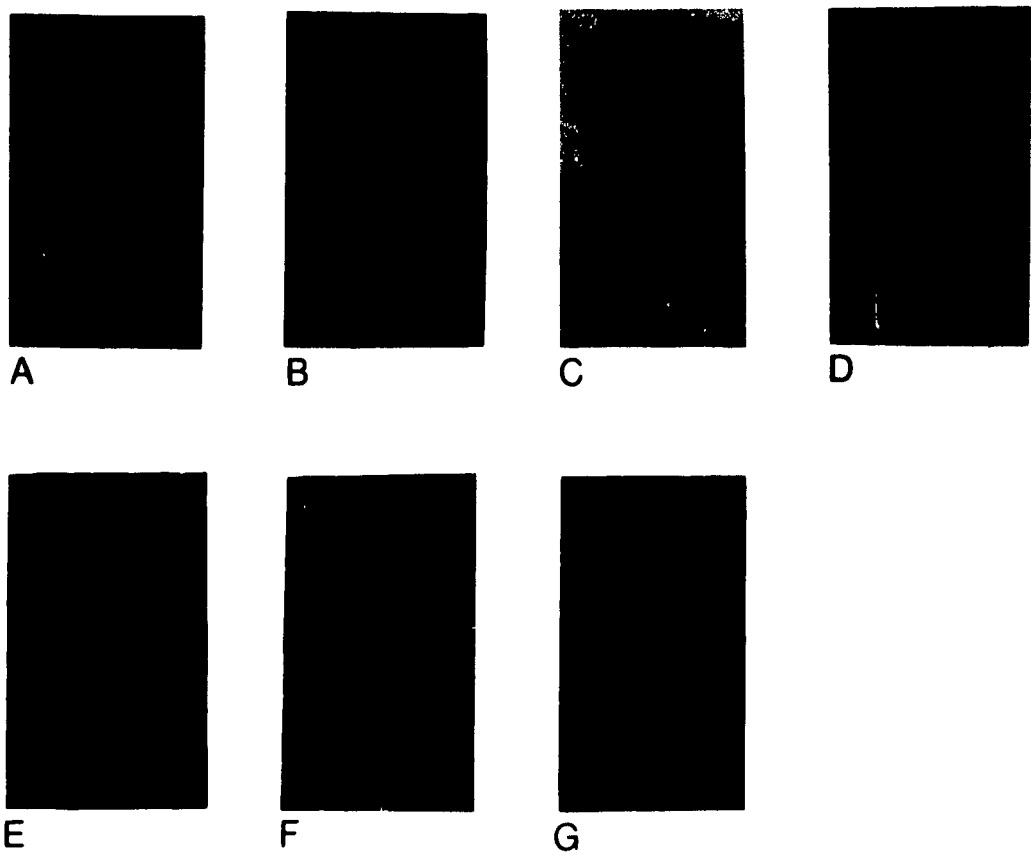

Figure 6. Panels A-D are original magnifications of treated S. epidermidis biofilms. Panels E-G present the bacterial morphology (x 1000 magnification) of similarly treated biofilms examined under monochromatic blue light microscopy. Panels A and E indicate the effect of exposure to peptone water for 24 hours and are representative of the viable control. Panels B and F indicate the effect of exposure to oleandomycin (45 µg/ml) in peptone water for 24 hours, and is representative of 34 antibiotics tested which had no demonstrable effect against S. epidermidis in the biofilm phase. Panels C and G indicate the superior efficacy of rifampin (20 µg/ml) in peptone water for 24 hours, showing predominant destruction of biofilm bacteria. Also clearly evident in panel C is the emergence of rifampin-resistant foci. Panel D represents the sterile control, showing the killing effect of 4 hours exposure to a 4% formol peptone solution.

4.1.2 Kinetics of rifampin activity. A decimal range of rifampin concentrations (100-0.01 µg/ml) was evaluated for the ability to produce inhibition and predominant killing of biofilms at hourly intervals up to 24 hours. Results are presented in Figure 7. At a concentration of 100 µg/ml, rifampin exhibited significant antimicrobial activity even when exposure was reduced to 6 hours. Conversely, with an exposure of 24 hours, rifampin demonstrated equivalent antimicrobial activity even when concentration was reduced to 0.01 µg/ml. A predominantly bactericidal effect (Figure 8) was noted with scattered foci of resistance (Figures 9 and 10).

There is a clear relationship between the concentration of rifampin and the time required for inhibition or kill. This seems to be a log-third order relationship and is not linear with respect to the log of concentration. This complex relationship is an unexpected finding which indicates that antibiotic action on S. epidermidis biofilms is not a simple process.

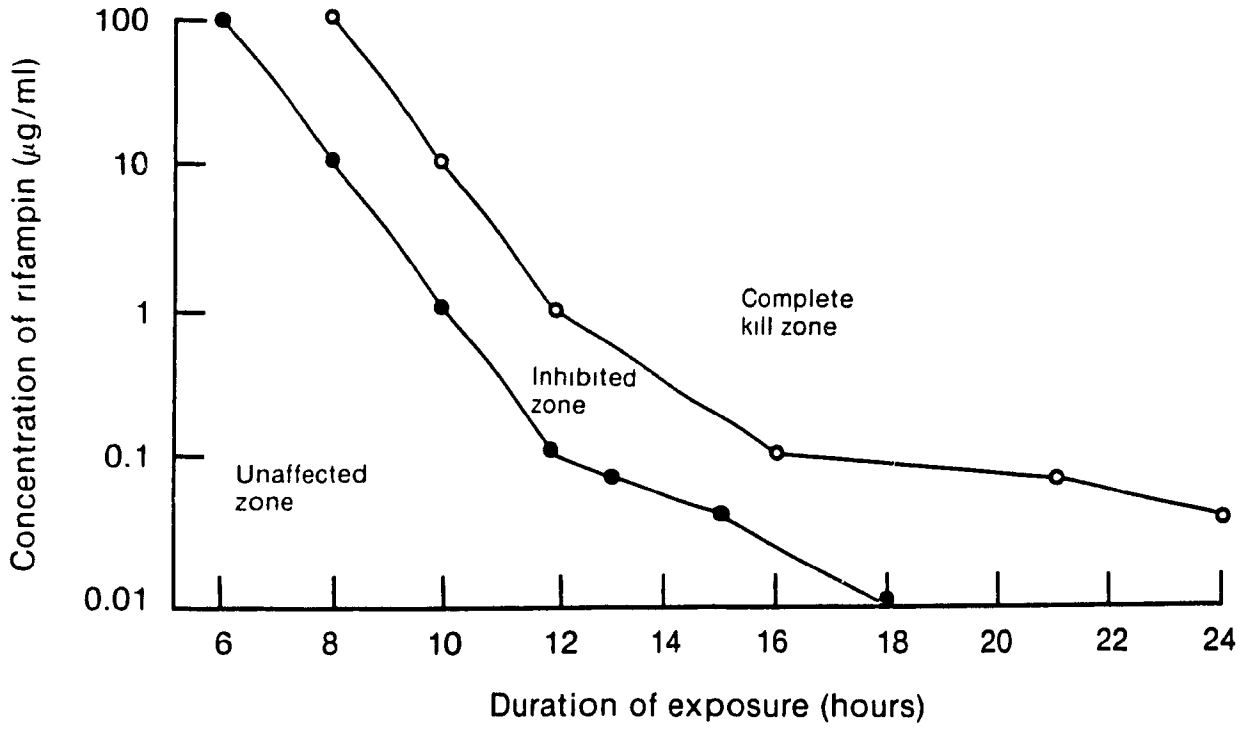

Figure 7. Rifampin activity presented as the log concentration-exposure time against S. epidermidis biofilms (peptone water diluent). The vertical axis is logarythmic.

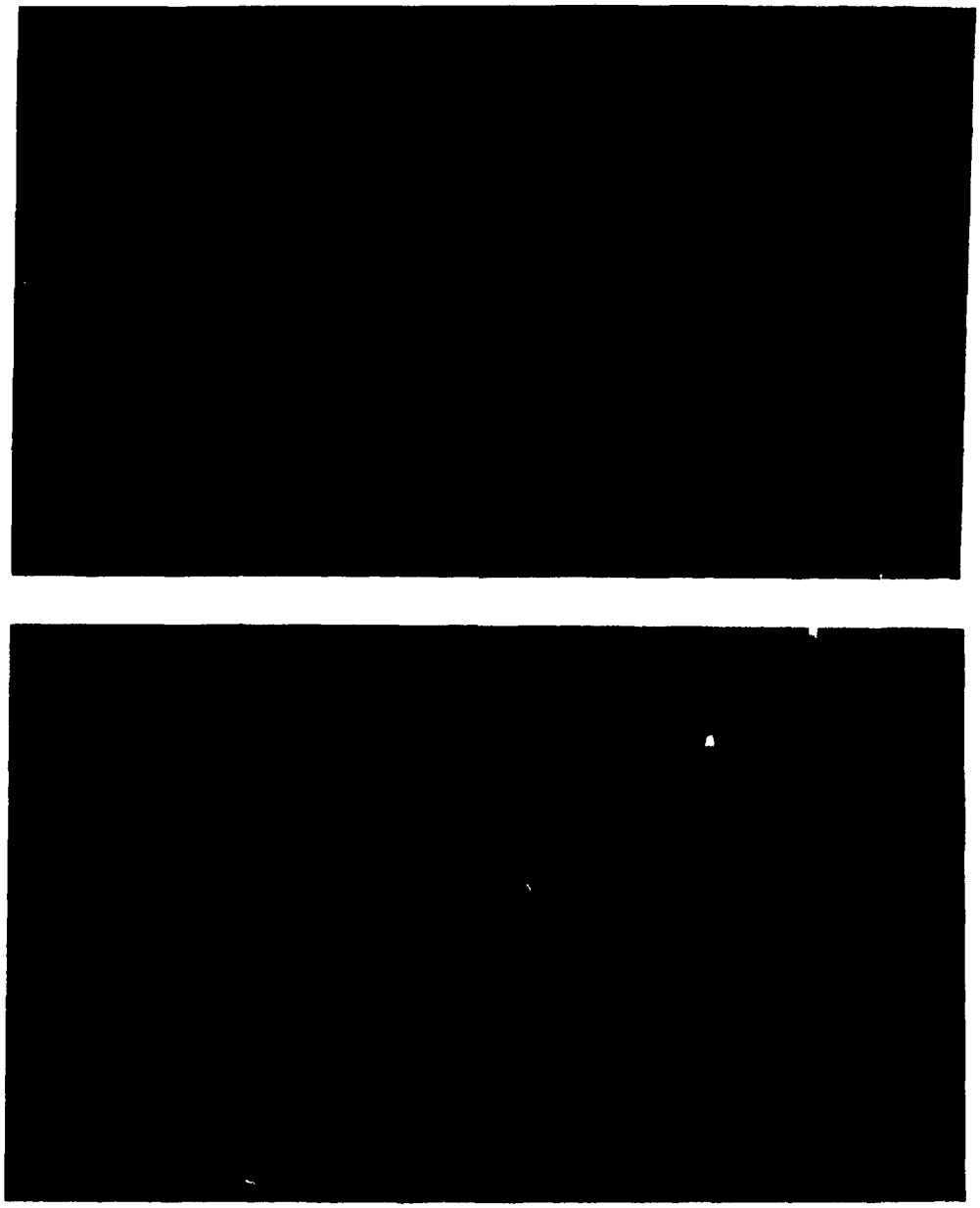

Figure 8. Light microscopy of representative S. epidermidis biofilms (original magnification x 1000). Top panel, Fully viable bacteria of an untreated biofilm, showing the density and uniformity of coccal forms. Bottom panel, Biofilm following 18 hours exposure to rifampin at 10 µg/ml in peptone water, demonstrating gross lysis, distortion, swelling and clumping of bacterial cells.

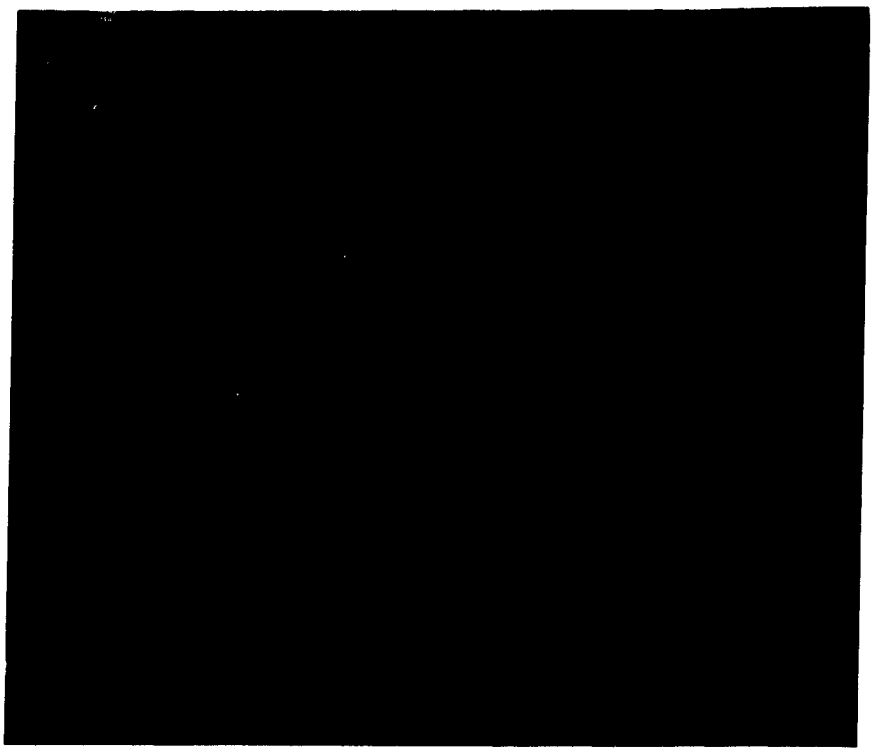

Figure 9. Phase contrast light microscopy by oil immersion of an S. epidermidis biofilm exposed to rifampin at 10 µg/ml in peptone water for 18 hours. Areas of clear blue background are representative of gross lysis of the biofilm. Pale blue/white coccal shapes are the remaining dead ghost cells of S. epidermidis. Bright spots indicate the emergence of rifampin-resistant S. epidermidis cells.

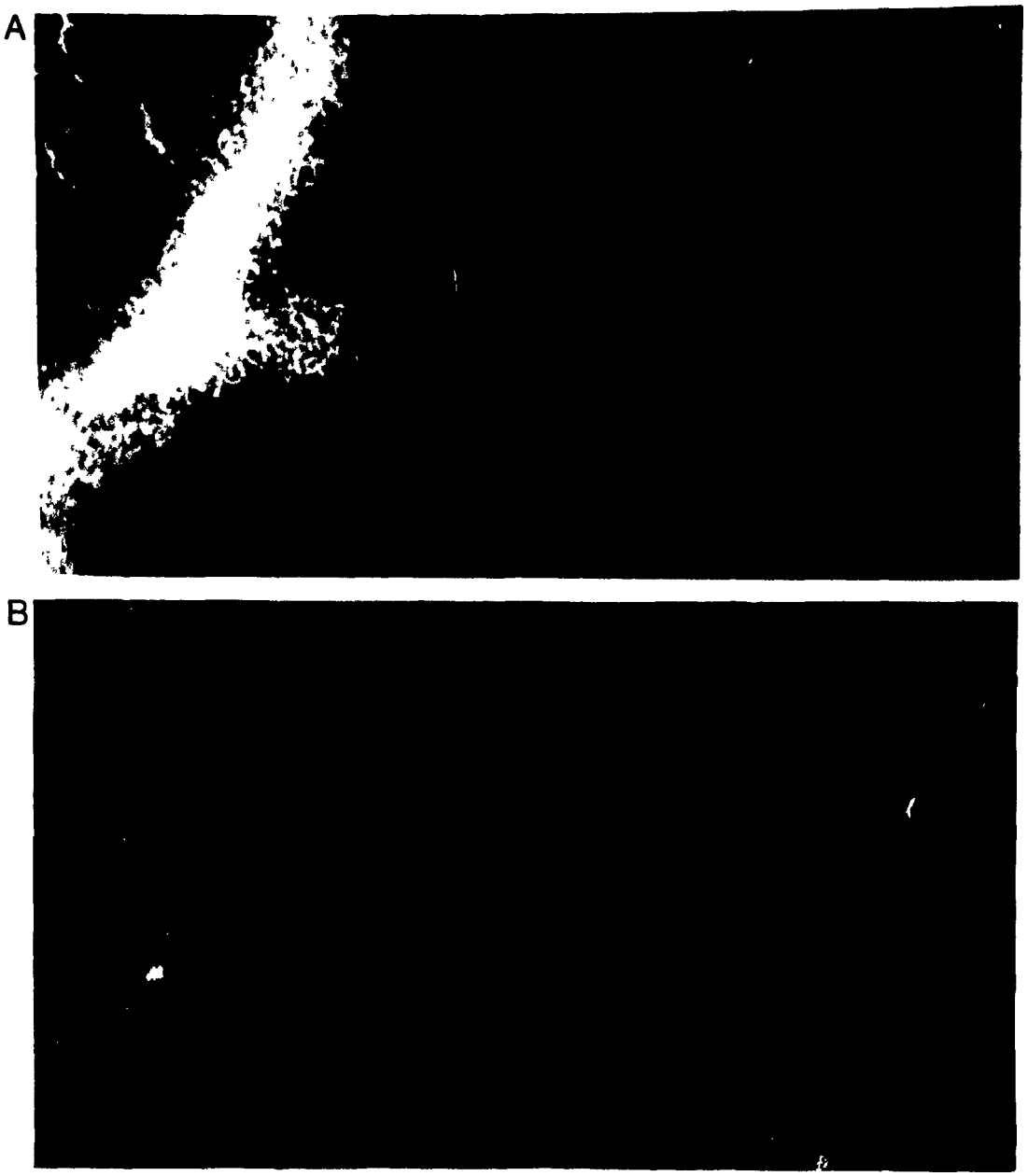

Figure 10. A and B represent the edge of a rifampin-resistant colony examined by phase contrast microscopy in which changes in refractive index are matched by changes in colour (Nomarski interference phase effect, original magnification x 750). From left to right in both panels: the bright rings demarcating the pink or red colony edge demonstrate the differing density of bacteria between the colony proper and the spreading bacterial growth; the bright spots are individual viable resistant S. epidermidis cells; the light blue and green areas represent dead ghost cells; and the solid dark blue and green areas indicate completely sterilized surfaces.

4.1.3 Antibiotic combinations. Rifampin alone (10 µg/ml) had significant antibacterial activity producing inhibition at 8 hours of exposure and predominant killing with minor foci of resistance at 10 hours (Table 2, left columns). The finding of the superior activity of rifampin, albeit incomplete, led to the determination of the outcome of rifampin activity in combination with other antibiotics. With peptone water as diluent, the efficacy of rifampin was evaluated in separate combinations with clindamycin, cloxacillin, fusidic acid, gentamicin, oxacillin, tetracycline and vancomycin upon the metabolic activity of S. epidermidis biofilms.

The effect of combinations with rifampin is seen in Table 2 (right columns). It is noted that clindamycin, fusidic acid, gentamicin and tetracycline clearly antagonized the antibacterial activity of rifampin. In contrast cloxacillin, oxacillin and vancomycin completed the activity of rifampin producing synergistic killing.

Table 2. Effects of antibiotics, singly or in combination with rifampin, on the exposure time (hours) required to produce inhibition (I) or killing (K) of S. epidermidis biofilms.

	Single agent		Combined with rifampin	
	I	K	I	K
Rifampin	8	10*	8	10*
Cloxacillin	>24	>24	8	12
Oxacillin	>24	>24	8	12
Vancomycin	>24	>24	10	18
Clindamycin	>24	>24	>24	>24
Fusidic acid	>24	>24	>24	>24
Gentamicin	>24	>24	>24	>24
Tetracycline	>24	>24	>24	>24

*Predominant killing with minor foci of resistance.
>24: no effect detected at 24 hours of exposure.
All antibiotics tested at a concentration of 10 µg/ml in peptone water.

4.1.4 Modulation of antibiotic activity by peritoneal dialysis solutions. The antimicrobial activity of antibiotics against S. epidermidis biofilms had been assessed using peptone water as a standard diluent. Replacing peptone water by peritoneal dialysis (PD) solutions produced a strikingly different outcome for several antibiotics. Table 3 lists the modifying effect of fresh and spent PD solutions on the antibiotic activity of clindamycin, gentamicin, rifampin, tetracycline and vancomycin. Neither fresh nor spent PD solutions produced

any change in the lack of activity of clindamycin, tetracycline and vancomycin upon the biofilm preparations. Gentamicin, which had been demonstrated inactive in peptone water, showed a weak reversible inhibitory effect in fresh and spent PD solutions after 18 hours of incubation. In marked contrast fresh PD solutions significantly enhanced the activity of rifampin producing inhibition at 4 hours and synergistic killing at 6 hours. Equally, spent PD fluid was clearly antagonistic to rifampin activity neutralizing these enhancements; inhibition was increased to 18 hours and no killing was observed even with 24 hours of antibiotic exposure.

Table 3. Effects of fresh and spent peritoneal dialysis solutions on antibiotic activity upon S. epidermidis biofilms: exposure time (hours) to produce inhibition (I) or killing (K).

Diluent:	Dialysis solution (1.5%) Fresh		Spent		Peptone water	
	I	K	I	K	I	K
Rifampin	4	6	18	>24	8	10*
Gentamicin	18	>24	18	>24	>24	>24
Clindamycin	>24	>24	>24	>24	>24	>24
Tetracycline	>24	>24	>24	>24	>24	>24
Vancomycin	>24	>24	>24	>24	>24	>24

*Predominant killing with minor foci of resistance.
>24: no change at the maximum exposure of 24 hours.
All antibiotics were tested at a concentration of 10 µg/ml.

Given the modulating effect of fresh and spent PD solutions on rifampin activity, the modulating potential of rifampin combinations using clindamycin, gentamicin, tetracycline and vancomycin was assessed (Table 4). With the exception of vancomycin there was clear antagonism demonstrated. Vancomycin produced minor prolongation of rifampin activity but paradoxically produced total killing in the presence of peptone water (Figure 11). As observed previously with rifampin alone, fresh PD solution accelerated the action of this antibiotic combination (rifampin and vancomycin) and spent PD fluid neutralized it.

Table 4. Effects of fresh and spent peritoneal dialysis solutions on the activity of rifampin antibiotic combinations against S. epidermidis biofilms: exposure time (hours) to produce inhibition (I) or killing (K).

Diluent:	Dialysis solutions (1.5%) Fresh		Spent		Peptone water	
	I	K	I	K	I	K
Rifampin	4	6	18	>24	8	10*
Rifampin + vancomycin	6	12	24	>24	10	18
Rifampin + gentamicin	>24	>24	>24	>24	>24	>24
Rifampin + tetracycline	24	>24	24	>24	>24	>24
Rifampin + clindamycin	>24	>24	>24	>24	>24	>24

*Predominant killing with minor foci of resistance.
>24: no change at the maximum exposure of 24 hours.
Rifampin concentration was 20 µg/ml; the concentration of the other antibiotics was 10 µg/ml.

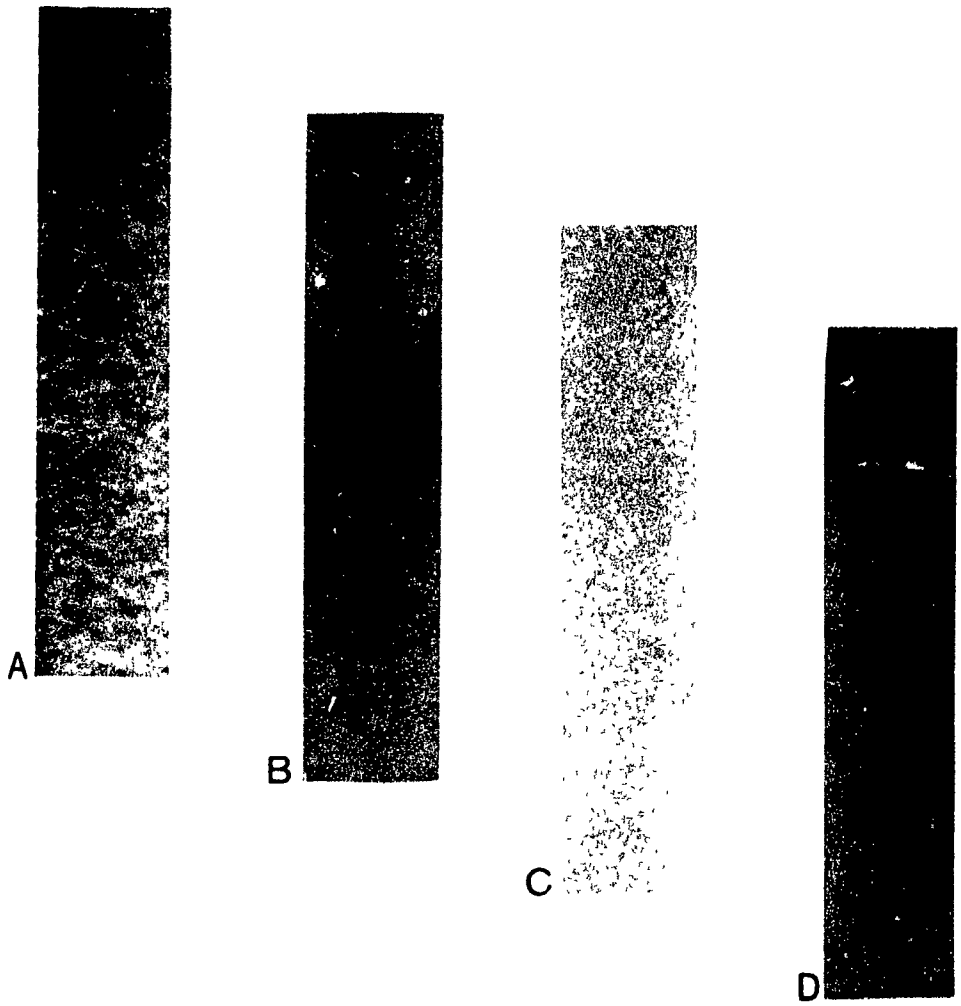

Figure 11. The progressive synergistic killing effect of rifampin and vancomycin (10 µg/ml each) in peptone water on standardized S. epidermidis biofilm preparations. Colour intensity of red formazan precipitate is an indication of bacterial viability. Panels A, B and C are respectively representative of 4, 8 and 12 hours exposure. Panel D represents 18 hours exposure and indicates a complete killing effect.

4.1.5 Concentration-time exposure kinetics of rifampin activity in various milieus. The modulating effect of fresh 1.5% PD solution on rifampin activity against S. epidermidis biofilm preparations was examined. Using a 10,000-fold range in rifampin concentration, the metabolic activity of biofilms was assessed at hourly intervals over a 24 hour period. It is noted that even at concentrations as high as 100 µg/ml and exposure time of 24 hours, using peptone water as a diluent, complete killing was not obtained (Figure 12, bottom panel). In marked contrast, rifampin demonstrated complete killing at all concentrations tested when in the presence of fresh PD solution (Figure 12, top panel).

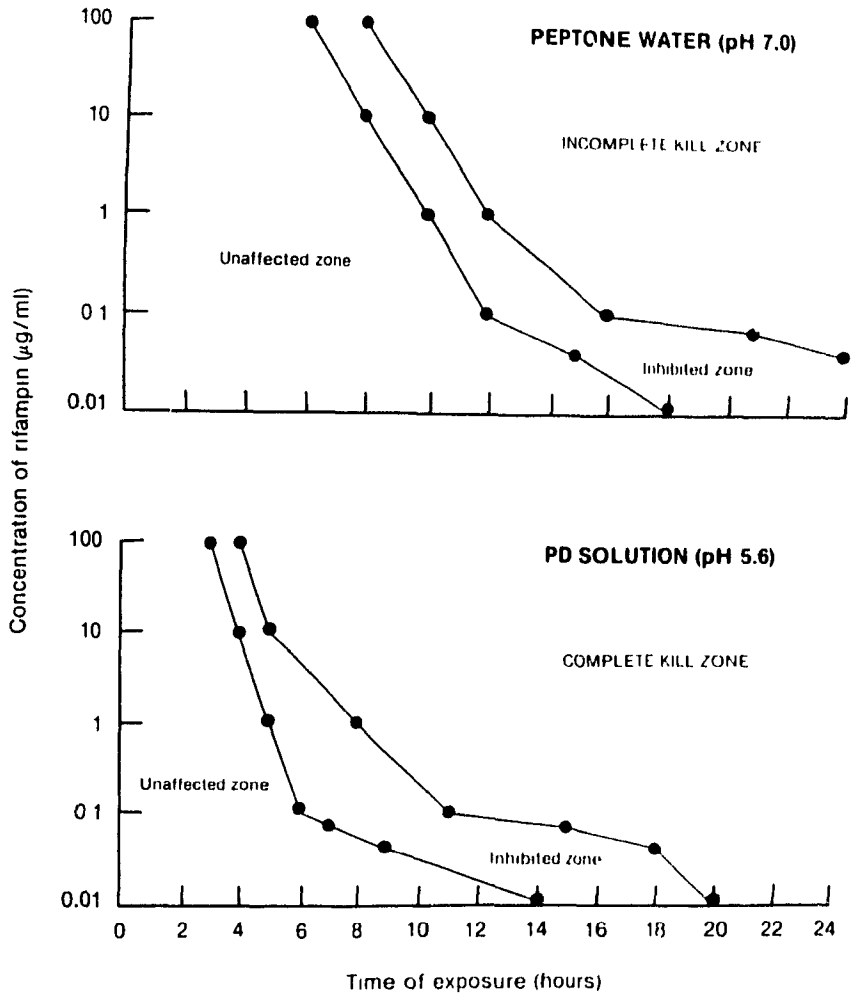

Figure 12. Concentration-time exposure kinetics of rifampin activity against standardized S. epidermidis biofilms in peptone water and fresh peritoneal dialysis solution (1.5% dextrose). Rifampin doses (vertical axis) over a 10^4-fold range are logarythmic.

4.1.6 Fresh versus spent peritoneal dialysis solutions. Some of the recognized differences between fresh and spent PD solutions were examined in an attempt to explain the differences in their modulating effects. It is evident that among the differences between fresh and spent PD solutions, the rise in pH and the reduction in dextrose concentration are physiologically significant. The effect of pH and dextrose concentration on the standardized activity of rifampin against S. epidermidis was assessed, contrasting the fluid phase (Table 5) of the bacteria with the biofilm preparation (Table 6). To examine the variation in pH and dextrose content of PD solution, fresh PD solutions over the range of 0.5, 1.5, 2.5 and 4.25% at both normal pH and buffered to pH 7 by the addition of HEPES were used. Data for 0.5% and 2.5% dextrose concentrations are not included in the text as there was no appreciable difference from those obtained with 1.5 and 4.25% which are the concentrations most frequently utilized clinically.

(a) Fluid phase.

The effect of pH and dextrose concentration on the qualitative assessment of the metabolic activity of S. epidermidis in fluid phase culture is noted Table 5. In peptone water the reduction of pH from 7 to 5 resulted in a progressive slowing of metabolism. Similarly in fresh PD solutions (1.5% and 4.25% dextrose concentration) a fall in pH from 7 to 5.6 and 5.2, respectively, produced a comparable change. It is evident that this range of dextrose concentration (0.5-4.25%) and corresponding

osmolarity (280-490 mOsm/kg) of the PD solutions tested is without effect and that the fluid phase metabolism of S. epidermidis is markedly pH dependent. Pooled spent PD fluid at pH 7 was a significant inhibitor of metabolic activity. None of the media or variables (pH, dextrose) tested affected the complete killing of fluid phase bacteria by rifampin at 10 µg/ml.

Table 5. Effects of media and pH on the metabolic activity and response to rifampin of fluid phase S. epidermidis following 18 hours exposure.*

Exposure media:	Media alone	Combined with rifampin (10 µg/ml)
Peptone water (pH 5)	+	0
Peptone water (pH 6)	++	0
Peptone water (pH 7)	+++	0
Fresh 1.5% PD solution (pH 5.6)	++	0
Fresh buffered 1.5% PD solution (pH 7)	+++	0
Fresh 4.25% PD solution (pH 5.2)	++	0
Fresh buffered 4.25% PD solution (pH 7)	+++	0
Spent 1.5% PD fluid (pH 7)	+	0

*Semi-quantitative grading of bacterial metabolic activity as indicated by red formazan precipitate 24 hours after the addition of aqueous TTC.
Buffering of PD solutions with HEPES.

(b) Biofilm phase

This range of tests was repeated for S. epidermidis biofilms but with the additional variable of recovery at pH 5 and 6 in addition to standard pH 7 (Table 6). The purpose was to determine the effect of prolonged pH change. Given that the pH of the exposure phase is highly significant and because of the possible therapeutic implications, the modulating effect of altering the pH during the recovery was explored.

At 4 hours exposure the pH of exposure and pH of recovery had no effect on the viability of the biofilms exposed to peptone water, PD solutions, fresh and spent. Rifampin in peptone water was without effect unless both exposure and recovery media were at pH of 5. Conversely the effects of rifampin in fresh PD solutions were enhanced by the reduced pH of the recovery media. Spent PD fluid neutralized the effect of rifampin at all pH tested.

At 18 hours exposure to peptone water as a control, the pH of exposure and the pH of recovery had no effect on viability. The killing activity of rifampin was enhanced by the pH of exposure, independent of the pH of recovery. Using fresh PD solutions the reduced pH of recovery altered the viability of the biofilms and similarly enhanced the effect of rifampin. Using spent PD fluid (pH 7) the neutralizing effect upon rifampin activity was diminished by reduction in pH of recovery. In summary the pH of exposure and the pH of recovery independently influenced the activity of rifampin against the S. epidermidis biofilms.

Table 6. Relationship of exposure media composition and recovery-indicator medium pH on the response of S. epidermidis biofilms to rifampin.

	pH of recovery-indicator medium		
	5	6	7
4 hours exposure			
Peptone water (pH 5)	V	V	V
" + rifampin	K	V	V
Peptone water (pH 6)	V	V	V
" + rifampin	V	V	V
Peptone water (pH 7)	V	V	V
" + rifampin	V	V	V
Fresh 1.5% PD solution (pH 5.6)	V	V	V
" + rifampin	K	K	I
Fresh buffered 1.5% PD solution (pH 7)	V	V	V
" + rifampin	K	I	I
Spent 1.5% PD fluid (pH 7)	V	V	V
" + rifampin	V	V	V
18 hours exposure			
Peptone water (pH 5)	V	V	V
" + rifampin	K	K	K
Peptone water (pH 6)	V	V	V
" + rifampin	K	K*	K*
Peptone water (pH 7)	V	V	V
" + rifampin	K*	K*	K*
Fresh 1.5% PD solution (pH 5.6)	K	I	I
" + rifampin	K	K	K
Fresh buffered 1.5% PD solution (pH 7)	K	I	I
" + rifampin	K	I	I
Spent 1.5% PD fluid (pH 7)	V	V	V
" + rifampin	I	I	V

Abbreviations are: V: viable, I: inhibited, K: killed, K*: predominant kill with minor foci of resistance. pH modification of PD solutions by HEPES buffer. For the sake of clarity results obtained in the presence of rifampin are slightly indented to the right of the columns.

4.1.7 Effects of drug additives to fresh peritoneal dialysis solutions on the metabolic activity of S. epidermidis biofilms. The effect of nine PD solution drug additives including calcitriol, desferrioxamine, furosemide, heparin, insulin, phosphatidylcholine, streptokinase, verapamil and urokinase on the metabolic activity of S. epidermidis biofilms was examined (Table 7, left columns). With the exception of furosemide and phosphatidylcholine, the additives were without any effect after 24 hours exposure. Furosemide and phosphatidylcholine showed a minor inhibitory effect at 18 hours exposure. This was a reversible phenomenon. The potential for interference of these additives on the activity of rifampin against S. epidermidis biofilms was also examined (Table 7, right columns). It was notable that only desferrioxamine, furosemide, phosphatidylcholine and streptokinase were inert and did not interfere with the activity of rifampin. In contrast, calcitriol, heparin, insulin, urokinase and verapamil brought about complete neutralization of rifampin activity at the tested concentrations.

The minor inhibitory effects of additives had been assessed using peptone water as a standard diluent. Replacing peptone water by PD solutions produced a different picture. The reversible inhibitory effect of furosemide and phosphatidylcholine was maintained using fresh PD solution but disappeared in spent PD fluid (data not shown). The lack of activity of the other additives was maintained in

both fresh and spent PD solutions (data not shown).

Table 7. Effects of peritoneal dialysis drug additives on the exposure time (hours) required to produce inhibition (I) or killing (K) of S. epidermidis biofilms, singly or in the presence of rifampin.

	Single agent		Combined with rifampin (10 μg)	
	I	K	I	K
Rifampin (10 μg)	8	10*	8	10*
Desferrioxamine (5 mg)	>24	>24	8	10*
Streptokinase (500 U)	>24	>24	8	10*
Furosemide (1 mg)	18	>24	8	12*
Phosphatidylcholine (0.15 mg)	18	>24	8	12*
Calcitriol (0.01 mg)	>24	>24	>24	>24
Heparin (10 U)	>24	>24	>24	>24
Insulin (0.03 U)	>24	>24	>24	>24
Urokinase (5 U)	>24	>24	>24	>24
Verapamil (0.05 mg)	>24	>24	>24	>24

*Predominant killing with minor foci of resistance.
>24: no effect detected at 24 hours of exposure.
Drug concentration per ml of peptone water diluent is indicated in parentheses.

4.1.8 Antimicrobial activity of different rifampin preparations and analogs against S. epidermidis biofilms. For the purpose of strict comparison, the two injectable rifampin preparations currently available (Ciba-Geigy, Merrell-Dow) were evaluated concurrently with the original

therapeutic formulation (Sigma) as well as other rifamycin formulations, including two recently developed analogs. All formulations were solubilized in dimethylformamide which has been demonstrated to have no interfering antimicrobial activity per se.

Using peptone water as the medium, inhibition of the biofilms required an exposure time of 10 hours for all six antibacterial agents at a concentration of 10 µg/ml (Table 8). The exposure time required for killing the bacterial biofilms was 12 hours for five of the agents but 18 hours of exposure was necessary for the rifamycin SV compound. Killing of the biofilms however was incomplete, with residual foci of antibiotic resistant cells (Figures 9 and 10). By contrast, only 0.001 µg/ml of the varying rifamycin compounds was required to produce both inhibition and killing at an exposure time of 18 hours for the fluid phase of this particular strain of S. epidermidis.

The modulating effects of fresh PD solution (1.5% dextrose concentration) upon antimicrobial activity was also examined (Table 8). Both clinically available forms of rifampin equally showed inhibition and killing at exposure time significantly reduced to 4 and 6 hours, respectively. It is significant to note that in the fresh PD solution environment, complete killing occurs. No metabolic activity was detected after 5 days of incubation of the antibiotic-free biofilms on the R-IM. The effects of fresh 1.5% PD solution on all six antibiotics showed equivalent shortening in exposure time required for

inhibition and killing. Killing was produced within 8 hours with the exception of rifamycin SV which required 10 hours. The complete killing obtained with all six antibiotics indicates uniform synergism of fresh PD solution on the activity of these rifamycins.

Using spent PD fluid as the environment yielded strikingly different results (Table 8). All six agents required an extension of exposure time of at least 18 hours to produce inhibition (24 hours for rifamycin SV). At 24 hours of exposure only the two rifampin analogs (CGPO29861, Ciba-Geigy, and rifapentine, Merrell-Dow) produced killing of the biofilms. Spent PD fluid clearly exerted a profound antagonistic effect on the antimicrobial activity of these rifamycins. It was conjectured that these antagonistic effects of spent PD fluid may be ascribed to protein content or increased alkalinity when compared to fresh PD solution. To test this conjecture, fresh 1.5% PD solution was modified by the addition of 0.05% foetal calf serum and the antimicrobial activity of the rifamycins was reassessed (Table 8). This addition of protein produced no alteration of the exposure time required for inhibition, with a minor increase in exposure time to 10 hours to produce complete killing. The sole exception was rifamycin SV which was inhibited by the addition of protein, no sterilization being evident with an exposure time of 24 hours. It is significant that supplementation of fresh 1.5% PD solution with a fivefold increase in foetal calf serum concentration (0.25%) did not alter the aforementioned results (data not

shown). Fresh 1.5% PD solution was also modified by the addition of HEPES buffer to pH 7 and antimicrobial activity of the rifamycins was reexamined. No prolongation of the exposure times required to produce inhibition was found, but significant antagonism of killing activity was noted. With the sole exception of the analog CGP029861, killing was not observed at 24 hours of exposure.

Table 8. Exposure time (hours) to rifampin compounds required for inhibition (I) or killing (K) of Staphylococcus epidermidis biofilms.

	Peptone water		1.5% peritoneal dialysis solution							
			Fresh		Spent		Fresh + FCS		Fresh + HEPES	
	I	K	I	K	I	K	I	K	I	K
Rifampin (C-G)	10	12*	4	6	18	>24	4	10	4	>24
Rifampin (M-D)	10	12*	4	6	18	>24	4	10	4	>24
Rifampin (S)	10	12*	4	8	18	>24	4	10	4	>24
Analog 1 (C-G)	10	12*	4	8	18	24	4	10	4	10
Analog 2 (M-D)	10	12*	4	8	18	24	4	10	4	>24
Rifamycin SV (S)	10	18*	8	10	24	>24	10	>24	8	>24

Abbreviations are: C-G: Ciba-Geigy, M-D: Merrell-Dow; S: Sigma; FCS: foetal calf serum.
HEPES buffering to pH 7.
Heat inactivated FCS at 0.05% concentration.
Antibiotics solubilized in dimethylformamide and adjusted in the various exposure media to a concentration of 10 µg/ml.
Analogs 1 and 2 are CGP029861 and rifapentine, respectively.
*Minor foci of resistance observed.

4.1.9 Investigation of rifampin-resistant S. epidermidis. Isolates of resistant foci which emerged from standardized S. epidermidis biofilm preparations following 18 hours exposure to rifampin (Ciba-Geigy, 10 µg/ml) in peptone water were established on solid agar media. Prolonged subcultures of this rifampin-resistant isolate of S. epidermidis indicated purity and non-reversion.

a) Fluid phase

The metabolic activity of the rifampin-resistant (RR) strain was assessed in various solutions, in the presence or absence of rifampin, and compared to the rifampin-sensitive (RS) parent strain (Table 9). The RR strain demonstrated comparable preservation of metabolic activity in all solutions, regardless of whether rifampin was present or not. The metabolic activity of both S. epidermidis strains in various preparations of peptone water and fresh PD solutions was clearly pH dependent and did not appear to be influenced by either protein or dextrose. In contrast, the reduction of metabolic activity observed for both strains in spent PD fluid was not a function of pH.

Table 9. Metabolic activity of rifampin-sensitive (RS) and rifampin-resistant (RR) S. epidermidis in the fluid phase following 18 hours exposure to various media with and without rifampin.*

	RS S. epidermidis		RR S. epidermidis	
	Media alone	Combined with rifampin (10 µg/ml)	Media alone	Combined with rifampin (10 µg/ml)
Peptone water (pH 5)	+	0	+	+
Peptone water (pH 6)	++	0	++	++
Peptone water (pH 7)	+++	0	+++	+++
Fresh 1.5% PD solution (pH 5.6)	++	0	++	++
Fresh buffered 1.5% PD solution (pH 7.0)	+++	0	+++	+++
Fresh 4.25% PD solution (pH 5.2)	++	0	++	++
Fresh buffered 4.25% PD solution (pH 7.0)	+++	0	+++	+++
Spent 1.5% PD solution (pH 7.0)	+	0	+	+

*Semi-quantitative grading of bacterial metabolic activity as indicated by red formazan precipitate 24 hours after the addition of aqueous TTC.
Fresh PD solutions were buffered with HEPES.

b) Biofilm phase

The RR isolate of S. epidermidis was used to form standardized biofilms as previously described for the parent strain. This RR biofilm was compared to that of the RS S. epidermidis parent strain for susceptibility to rifampin, vancomycin and the combination. Each biofilm preparation was exposed to the antimicrobials in the standard peptone water milieu as well as in fresh and spent PD solutions for a period of 18 hours. The results of this study are presented in Table 10.

To the exception of rifampin alone, both the RR and RS S. epidermidis biofilms when exposed in peptone water demonstrated no differences in susceptibility to the antimicrobials tested. The RR strain remained fully resistant in the biofilm phase to rifampin (10 µg/ml) after 18 hours exposure. Additionally, as for the parent strain, this biofilm could be killed with the combination of rifampin and vancomycin (10 µg/ml each). This demonstrated that the RR biofilm shared similar properties to that of the emerging resistant colonies seen to develop in the parent strain following exposure to rifampin.

Changing the exposure media to fresh 1.5% PD solution produced a marked effect on the RR biofilm as compared to the RS parent strain. In the absence of antimicrobial agents, the RR biofilm was transiently inhibited by this media. The addition of vancomycin (10 µg/ml) synergistically inhibited the RR biofilm in fresh PD solution as compared to the parent strain. In contrast to

the efficacy of antimicrobials in peptone water, rifampin or vancomycin in fresh PD solution could also transiently inhibit the RR strain. Whether the observed synergistic effect of rifampin and vancomycin separately in fresh PD solution is attributable to the combination of antimicrobials and exposure media or is solely a factor of fresh 1.5% PD solution remains unclear.

As for the parent RS strain, all antimicrobials in spent 1.5% PD fluid were ineffective against the RR S. epidermidis biofilm. This suggests that the observed effect of fresh PD solution on RR biofilms was not attributable to dextrose but more possibly due to pH.

Table 10. Antimicrobial susceptibility of rifampin-sensitive (RS) and rifampin-resistant (RR) S. epidermidis biofilm preparations in various media following 18 hours exposure.

	Exposure media					
	Peptone water		Dialysis solutions (1.5%) Fresh		Spent	
	RS	RR	RS	RR	RS	RR
Antimicrobials						
Rifampin	(-)	+	-	(+)	(+)	+
Vancomycin	+	+	+	(+)	+	+
Rifampin + vancomycin	-	-	-	-	+	+
Control media	+	+	+	(+)	+	+

The bacterial biofilms consisted of 18 hour preparations on glass surfaces. Results are expressed as:
+ : viable, full metabolic activity.
(+): viable, transient metabolic inhibition.
(-): predominantly non viable, minor foci of resistance.
- : non viable, bactericidal effect.
Both strains (RS and RR) of S. epidermidis biofilms were sterilized within 3 hours with 4% formol saline.
The concentration of each antibiotic was 10 µg/ml.

4.1.10 Antimicrobial activity of chemical agents against S. epidermidis biofilms. A number of disinfecting agents of different classes including the aldehyde, biguanide, halide and peroxide groups together with a chlorous acid/chlorine dioxide generating system (RenNew-P formulations) were tested at customary working strength. The relative times required to sterilize standardized S. epidermidis biofilms by these agents are presented in Table 11.

Table 11. Sterilization of S. epidermidis biofilms by RenNew-P and common disinfecting agents.

Test formulations	Exposure time at 22°C for sterilization
RenNewP-1	5 sec
Amuchina (50%)	5 sec
Chlorhexidine (0.05%)	5 sec
Dakin's solution (0.5%)	5 sec
Povidone-iodine (10%)	5 sec
RenNewP-2	45 sec
RenNewP-3	3 min
RenNewP-4	10 min
Hydrogen peroxide (3%)	10 min
Formol saline (4%)	3 hr

Activity of RenNewP-1 against S. epidermidis biofilms. Care of the connection between the implanted CAPD catheter

and the external tubing is demanding and techniques are continuously being modified. In this clinical context, the activity of disinfecting agents was further examined to determine the effects of contamination or admixture with PD solutions. RenNewP-1 was the most active formulation of the RenNew-P compounds and the effects of dilution and diluents were examined (Table 12). The dilutions varied from 1:2 to 1:10 and the diluents comprised normal saline, fresh PD solution and peptone-saline. Four percent formol saline, a known potent environmental disinfectant of considerable toxicity, was used as a reference. Progressive dilutions resulted in an exponential prolongation of sterilization time. PD solution (4.25% dextrose concentration) markedly enhanced the antimicrobial activity but in a non-uniform manner, and the mechanism remains to be explored.

Table 12. Effects of dilution and diluents on the sterilization of S. epidermidis biofilms by RenNewP-1 and formol saline.*

	RenNewP-1			Formol saline		
Diluents**	NS	PDS	PS	NS	PDS	PS
Dilutions						
1/2	15 sec	5 sec	1 min	8 hr	6 hr	8 hr
1/3	30 sec	1 min	3 min	12 hr	6 hr	12 hr
1/5	5 min	5 min	1 hr	18 hr	24 hr	24 hr
1/7	15 min	7 min	4 hr	24 hr	24 hr	>24 hr
1/10	1 hr	10 min	24 hr	24 hr	24 hr	>24 hr

*The results are expressed as the minimal time of exposure at 22°C to effect sterilization of the biofilms.
**NS: normal saline; PDS: peritoneal dialysis solution (Inpersol 4.25%, Abbott Laboratories, Canada); and PS: peptone-saline.

The ability of protein to impair the activity of other disinfecting agents used in CAPD practice was also examined. The chosen parameter was the minimum concentration of agent required to sterilize the biofilms after 4 hours exposure (Table 13). The sterilizing activity of RenNewP-1 and Amuchina was neutralized by the presence of peptone at dilutions of 1:10 and 1:7, respectively. In this test system, the sterilizing effect of chlorhexidine and povidone-iodine was unaffected at dilutions of 1:15, the greatest dilution tested. Additionally, spent PD fluid was shown to completely antagonize the antimicrobial activity of RenNewP-1 up to 24 hours exposure (data not shown) (Appendix 3.B.8,12,15).

Table 13. Effect of protein on the sterilization of S. epidermidis biofilms by RenNewP-1 and common disinfecting agents.*

	Dilutions with peptone-saline					
	1:2	1:3	1:5	1:7	1:10	1:15
RenNewP-1	S	S	S	S	V	V
Amuchina 50%	S	S	S	V	V	V
Chlorhexidine (0.05%)	S	S	S	S	S	S
Povidone-iodine (10%)	S	S	S	S	S	S

*Results indicate the status of biofilm bacteria as V (viable) and S (sterilized) after 4 hours exposure at 22°C.

4.2 Peritoneal Catheter-Associated *Staphylococcus epidermidis* Preparations in the Mouse: Response to Rifampin

A mouse model of peritoneal catheter associated *S. epidermidis* biofilm infection was developed in the mouse to confirm the results obtained *in vitro* with rifampin against standardized *S. epidermidis* biofilm preparations. For this purpose a custom-made segment of the standard clinical CAPD catheter was used in the mouse (Figure 2A).

To ensure uniform biofilm preparations in all animals, *S. epidermidis* biofilms of appropriate age were formed on the catheter segments *in vitro* before their implantation in the peritoneal cavity of the mice. Controls consisted of the catheter alone and transiently dipped in fluid phase bacteria. A first experiment verified the stability of the implanted catheter/biofilm-complex. One and two weeks after implantation, bacterial recoveries and inflammatory response were correlated. No spontaneous clearance of the biofilm occurred on the catheter surface, yet infection did not propagate to the peritoneal cavity (Table 14). A local transient inflammatory response was observed during the first week following implantation of the combined catheter and bacteria, either in the fluid or the biofilm phase. No signs of a systemic inflammatory response were found (Table 15).

Table 14. Bacterial recoveries following the implantation of various S. epidermidis peritoneal catheter preparations.*

	Sites of bacterial recovery					
	Peritoneal washing		Catheter rinse (TSB/TTC)		Catheter (TSB/TTC)	
	1 wk	2 wk	1 wk	2 wk	1 wk	2 wk
Catheter alone	-	-	-	-	-	-
Catheter dipped in 10^8 CFU	-	-	-	-	-	-
Catheter and 1-day biofilm	-	-	+	+	+	+
Catheter and 2-day biofilm	-	-	+	+	+	+
Catheter and 3-day biofilm	-	-	+	+	+	+

*Results were obtained in normal C57BL/6 female inbred mice at 7 and 8 weeks of age.
TSB/TTC: a solution of tryptone soya broth and 2,3,5 triphenyltetrazolium chloride.
Peritoneal washings were plated on defibrinated horse blood agar.
All recovered specimens were incubated at 37°C for 5 days.
Results are expressed as:
-: no bacterial recovery.
+: recovery of S. epidermidis.

Table 15. Inflammatory response following the implantation of various S. epidermidis peritoneal catheter preparations.*

	Peritoneal washing				Peripheral blood			
	Leukocytes x 10^4/ml		Polys %		Leukocytes x 10^3/ml		Polys %	
	1 wk	2 wk	1 wk	2 wk	1 wk	2 wk	1 wk	2 wk
Catheter alone	20	21	5	4	3.1	9.2	9	6
Catheter dipped in 10^8 CFU	21	19	27	4	5.3	10.6	12	7
Catheter and 1-day biofilm	28	27	41	4	5.1	7.4	8	11
Catheter and 2-day biofilm	30	28	39	2	6.3	8.4	6	3
Catheter and 3-day biofilm	31	31	41	3	4.2	10.5	7	8

*Determinations performed in normal C57BL/6 female inbred mice at 7-8 weeks of age.
Results are expressed as median values in groups of 5 to 8 animals.

The following experiment addressed the response to rifampin, vancomycin, and the combination on the 3-day biofilm/catheter-complex in the mouse. The antibiotics were administered daily i.p. through a transcutaneous injection directly into the catheter lumen. The 7-day treatment period was commenced one week after the surgical implantation procedure. Results of the concurrent assessment of bacterial growth and inflammatory response are

presented in Tables 16 and 17, respectively.

Untreated animals demonstrated persistence of adherent S. epidermidis biofilm/catheter-complex without evidence of bacterial dissemination. In the control animals with catheters alone, the daily catheter manipulation and antibiotic administration did not lead to i.p. contamination. In accordance with the in vitro predictions, clearance of the biofilm was obtained only with rifampin. However, rifampin in this in vivo model provided total kill. Again in accordance with the in vitro results, vancomycin was inferior to rifampin with 3 of 5 animals displaying persistent biofilms at the time of sacrifice.

Antibiotic administration induced a striking local inflammatory response in all animals with catheter/biofilm-complexes. This was accompanied by a systemic inflammatory response, particularly in the rifampin-treated animals. An explanation for the observed inflammatory response to therapy is the release from the severely damaged biofilms of bacterial products with chemcattractant properties. These observations require further investigation.

Table 16. Effect of 7 days of antibiotic treatment on the bacterial growth of catheter adherent S. epidermidis biofilms.*

	Sites of bacterial recovery					
	Peritoneal washing		Catheter rinse (TSB/TTC)		Catheter (TSB/TTC)	
	C	C/B	C	C/B	C	C/B
No drug	-	-	-	+	-	+
Rifampin	-	-	-	-	-	-
Vancomycin	-	-	-	-	-	+(3/5)
Rifampin + vancomycin	-	-	-	-	-	-

*Measurements obtained in groups of 5 normal C57BL/6 female inbred mice 2 weeks after the peritoneal implantation of a catheter adherent S. epidermidis biofilm preparation. The 3-day biofilm was developed in vitro under standard conditions. Antibiotics were administered i.p. directly through the catheter lumen at 15 mg/kg/day for 7 consecutive days prior to sacrifice and assessment.
TSB/TTC: a solution of tryptone soya broth and 2,3,5 triphenyltetrazolium chloride.
C: catheter alone.
C/B: catheter/biofilm-complex.
All recovered specimens were incubated at 37°C for 5 days.
Results are expressed as:
-: no bacterial recovery.
+: recovery of S. epidermidis.

Table 17. Inflammatory response following 7 days of antibiotic treatment of catheter adherent S. epidermidis biofilms.*

	Peritoneal washing				Peripheral blood			
	Leukocytes x 10^4/ml		Polys %		Leukocytes x 10^3/ml		Polys %	
	C	C/B	C	C/B	C	C/B	C	C/B
No drug	21	31	4	3	9.2	10.5	6	8
Rifampin	20	393	4	30	5.9	12.9	5	30
Vancomycin	21	401	2	32	9.7	10.5	3	9
Rifampin + vancomycin	23	410	3	31	9.0	8.1	6	19

*Determinations performed in normal C57BL/6 female inbred mice at 7-8 weeks of age under the experimental conditions described in the legend to Table 16.
C: catheter alone.
C/B: catheter with adherent 3-day S. epidermidis biofilm.
Results are expressed as median values.

4.3 Effect of Drug Additives to Peritoneal Dialysis Solutions on the Viability and Function of Mouse Peritoneal Cells

4.3.1 Characteristics of the mouse model of chronic renal failure. In accordance with previous assessments in mice with renal failure of 6 weeks' duration (77,78), this study revealed evidence of marked retention of nitrogenous products, severe anemia and significant growth retardation (Table 18). The renal failure attenuated the early phase of the *in vivo* inflammatory response (predominantly polymorphs at 18 hours) whereas it had no effect on the later phase (predominantly macrophages at 3 days) (Table 19). It is notable that peritoneal cell populations were not affected by the surgery as shown in the sham-operated animals (Table 19).

Table 18. Plasma biochemistry, hematology and body weight of mice.[a,b]

	Normal	Sham surgery	Renal failure
Blood urea nitrogen, mg/dl	19.8±5.4(50)	26.3±6.8(35)	114.9±34.5[c](37)
Plasma creatinine, mg/dl	0.1±0.1(14)	0.2±0.1(8)	0.7±0.3[c](21)
Hemoglobin, g/dl	13.1±0.8(28)	13.0±0.7(30)	7.7±1.1[c](25)
Body weight, g	23.4±1.8(34)	22.1±1.5(31)	20.9±1.9[c](34)

[a]Results are expressed as mean±SD.
[b]Number of animals examined in each group is indicated in parentheses.
[c]Significant difference ($p<0.05$) between controls and renal failure mice.

Table 19. Characteristics of recovered mouse peritoneal cell populations.[a]

	Normal	Sham surgery	Renal failure
Resident cells			
Number of animals	24	12	16
Total number x 10^6/ml	0.8±0.3	0.6±0.2	0.8±0.4
Differential count, %			
Macrophages	49.9±8.2	61.1±11.4	47.5±13.6
Lymphocytes	45.8±8.9	36.0±10.0	50.6±13.6
Polymorphs	2.0±2.4	2.5±3.4	1.4±1.4
Mast cells	2.2±1.6	0.3±0.9	0.5±0.9
18-hour inflammatory exudate[b]			
Number of animals	13	7	14
Total number x 10^6/ml	2.6±1.0	2.6±1.2	1.5±0.8[d]
Differential count, %			
Macrophages	30.6±8.9	35.3±5.5	36.4±7.7
Lymphocytes	12.8±9.6	11.0±7.7	8.8±5.7
Polymorphs	56.5±12.6	53.6±12.5	54.8±10.8
Mast cells	0±0	0±0	0±0
3-day inflammatory exudate[c]			
Number of animals	26	23	21
Total number x 10^6/ml	2.3±0.7	2.4±1.1	2.4±1.0
Differential count, %			
Macrophages	74.8±8.1	73.7±8.2	70.4±7.1
Lymphocytes	16.8±6.7	16.1±6.7	19.6±6.4
Polymorphs	8.8±4.3	10.7±3.7	9.6±4.3
Mast cells	0.4±0.9	0.0±0.2	0.1±0.2

[a]Results are expressed as mean±SD.
[b,c]Peritoneal cell populations used in cytotoxicity and functional assays, respectively.
[d]Significant difference ($p<0.05$) between controls and renal failure mice.

4.3.2 Cytotoxicity of drug additives to peritoneal dialysis solutions towards mouse peritoneal cells. A cytotoxic effect of PD solutions on peritoneal cells was observed equally in the three animal groups. The cytotoxicity was more marked for resident than for elicited cells (Figures 13 and 14). In all instances the degree of cytotoxicity correlated with the concentration of dextrose of the PD solutions. The nine drug additives tested did not demonstrate any significant direct toxicity (Table 20, left columns). When the drug additives were combined with 4.25% PD solution, no further increase in cytotoxicity was observed, to the exception of the combination of phosphatidylcholine which was extremely cytotoxic (Table 20, right columns).

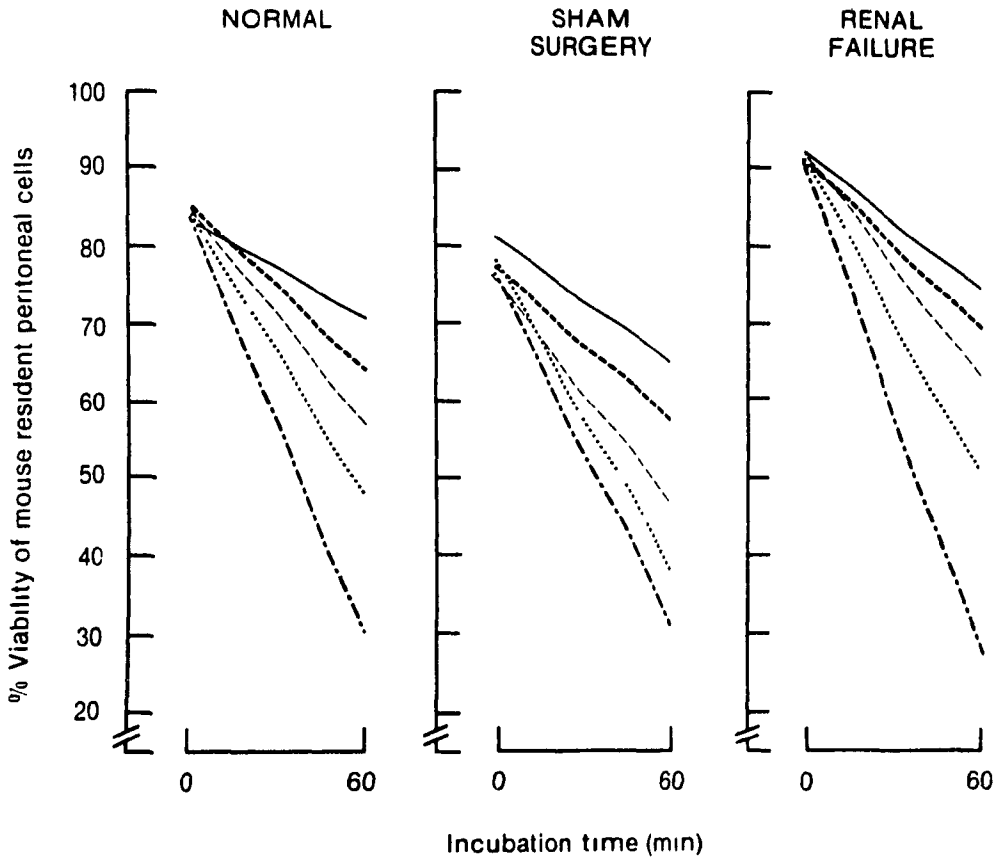

Figure 13. Cytotoxicity of PD solutions towards mouse resident peritoneal cells compared to PBS control (solid line). Mean percent viability of cells obtained from normal, sham-operated and renal failure animals are shown at 0 and 60 min of incubation. Dextrose concentrations of the PD solutions are 0.5% (---), 1.5% (---), 2.5% (•••) and 4.25% (•-•-).

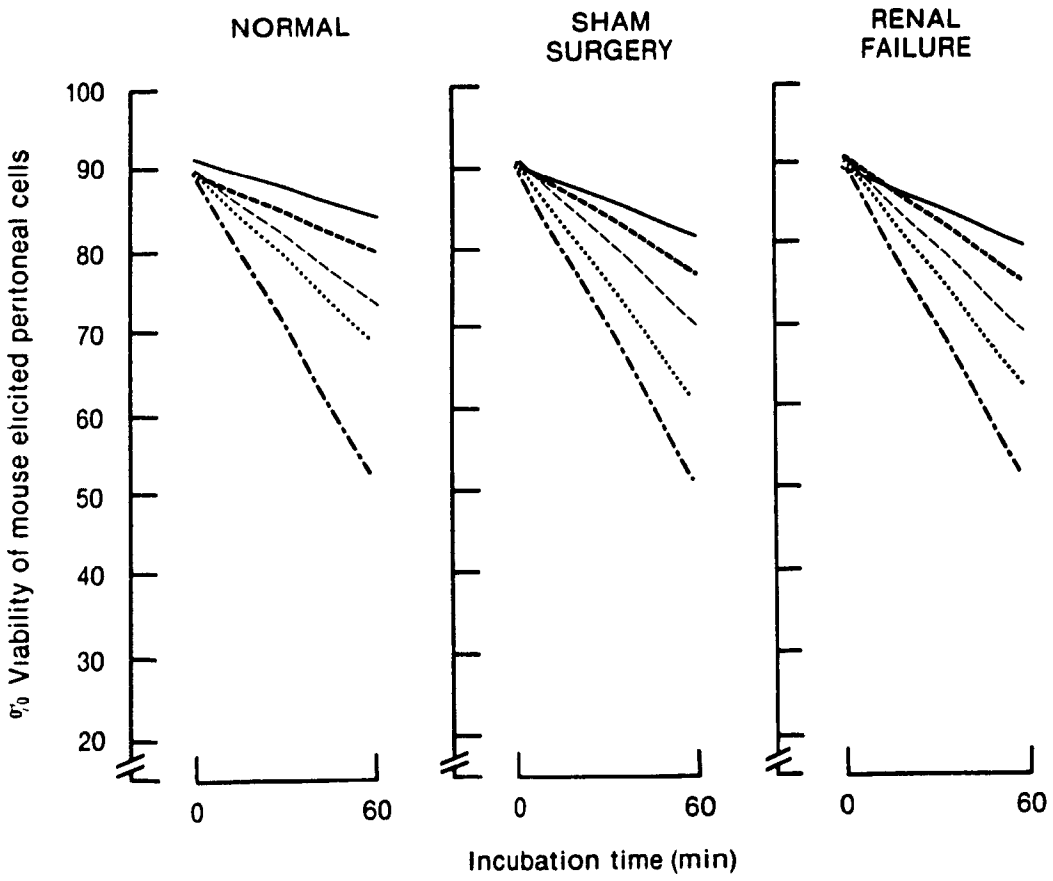

Figure 14. Cytotoxicity of PD solutions towards mouse elicited peritoneal cells compared to PBS control (solid line). Mean percent viability of cells obtained from normal, sham-operated and renal failure animals are shown at 0 and 60 min of incubation. Dextrose concentrations of the PD solutions are 0.5% (---), 1.5% (---), 2.5% (•••) and 4.25% (•-•-). Peritoneal cells were harvested 18 hours after i.p. injection of 3% Brewer's thioglycolate broth.

Table 20. Effect of drug additives to peritoneal dialysis solutions on the viability of normal mouse resident peritoneal cells.[a]

Nature of solution:	Control (PBS)		PD 4.25%	
Drug treatment:[b]	-	+	-	+
Calcitriol (0.01 mg)	90	91	69	83
Desferrioxamine (5 mg)	84	75	64	57
Furosemide (1 mg)	85	79	58	43
Heparin (10 U)	92	92	67	67
Insulin (0.03 U)	95	90	60	49
Phosphatidylcholine (0.15 mg)	84	82	56	6
Streptokinase (500 U)	86	79	58	76
Urokinase (5 U)	95	94	54	52
Verapamil (0.05 mg)	83	67	61	63

[a]Results are expressed as percent cell viability following 60 minutes exposure.
[b]Concentrations (per ml) of drug additives are indicated in parentheses.

4.3.3 Functional impairment of mouse peritoneal cells induced by drug additives to peritoneal dialysis solutions. Marked functional impairment of normal mouse macrophages was observed after incubation with 4.25% PD solution (Figure 15). In general, the drug additives when tested alone did not adversely affect macrophage function, with the exception of phosphatidylcholine (Figure 16). The combination of the drug additives separately to 4.25% PD solution strikingly affected cell function with complete inhibition of hydrogen peroxide production observed in all combinations (data not shown).

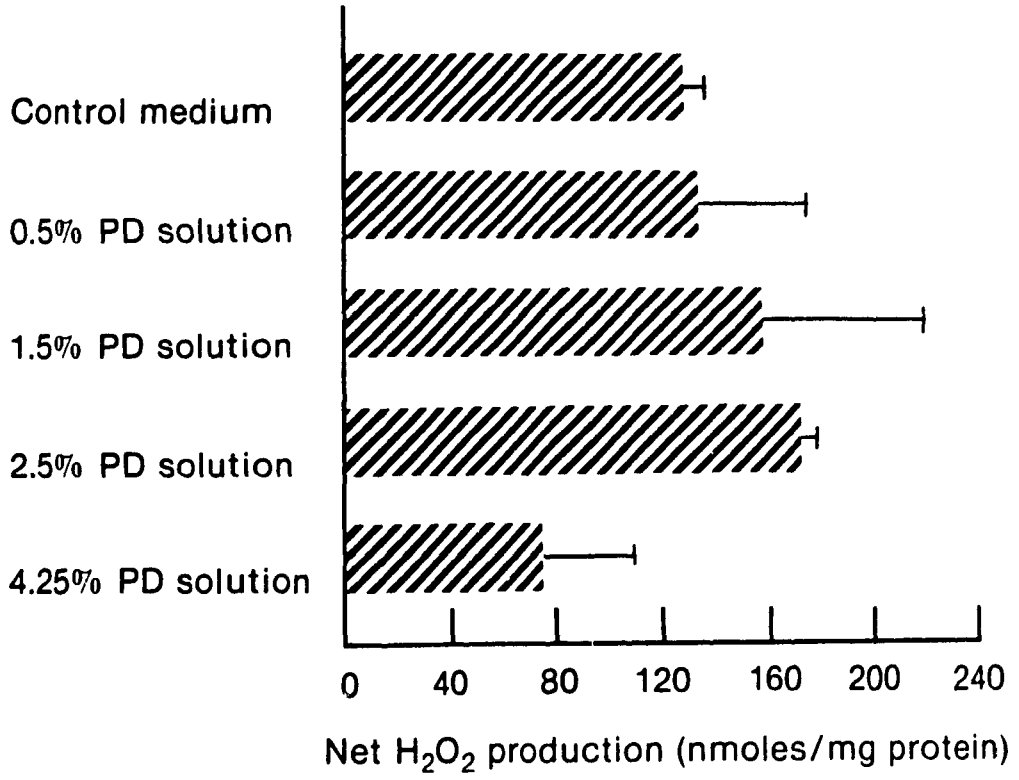

Figure 15. Influence of PD solutions of various dextrose concentrations on hydrogen peroxide (H_2O_2) production by normal mouse peritoneal macrophages compared to medium control (upper bar). Macrophages were obtained 3 days after i.p. injection of 3% Brewer's thioglycolate broth. Adherent macrophages were incubated for 60 minutes with the various test solutions prior to being stimulated with phorbol myristate acetate. Results are expressed as net H_2O_2 production, in nmoles/mg protein.

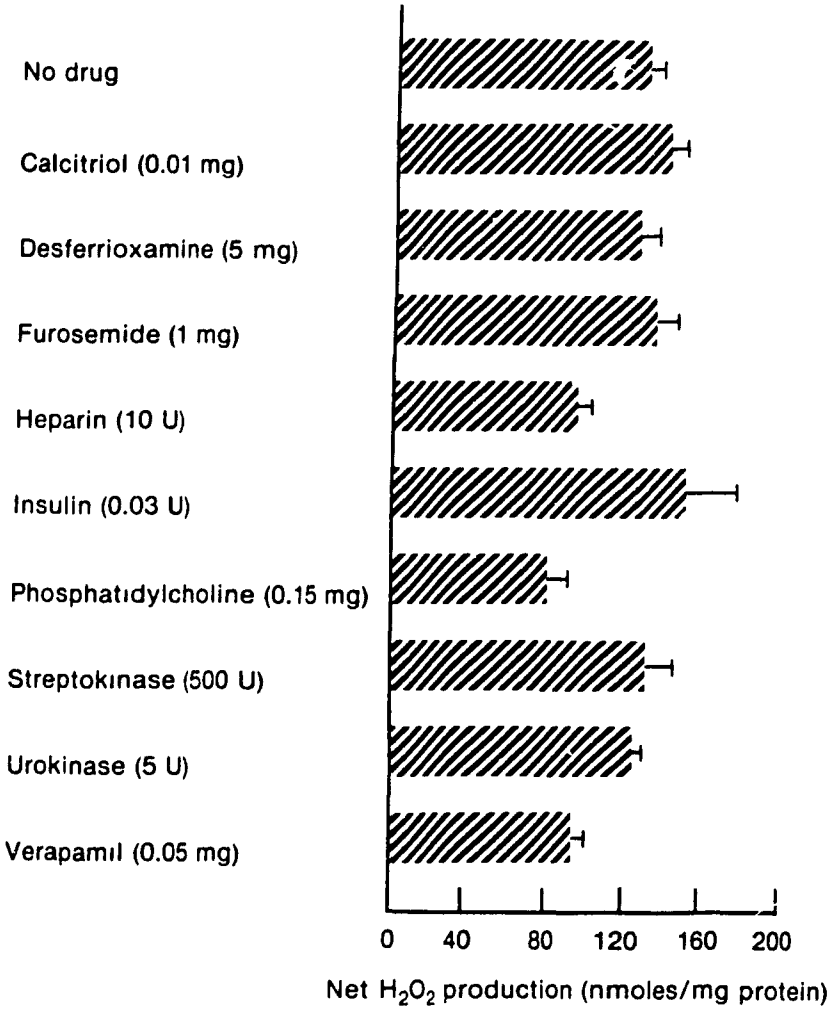

Figure 16. Influence of various drug additives to PD solutions on hydrogen peroxide (H_2O_2) production by normal mouse peritoneal macrophages compared to medium alone (upper bar). Macrophages were obtained 3 days after i.p. injection of 3% Brewer's thioglycolate broth. The drug concentrations are at the upper end of the range recommended for CAPD patients. Adherent macrophages were incubated for 60 minutes with the various test solutions prior to being stimulated with phorbol myristate acetate. Results are expressed as net H_2O_2 production, in nmoles/mg protein.

CHAPTER 5

DISCUSSION

The preceding investigations accomplished the initial thesis objectives. To be successful in achieving these aims, I had first to develop a series of novel methods to meet the technical challenges: 1) to standardize S. epidermidis biofilm preparations in vitro and in vivo; 2) to develop a technique to allow assessment of metabolic activity of biofilm bacteria in situ; and 3) to apply these to test the activity of antibiotics/disinfectants and explore the modulating effects on antimicrobial action of milieu, and of the addition of various therapeutic agents utilizing time dose kinetic studies on bacterial function.

It was demonstrated that standardized S. epidermidis biofilms together with a recovery-indicator medium can be readily used to evaluate antimicrobial activity. Current methods of evaluating bacterial biofilms utilize scanning electron microscopy, culture of biofilm fragments or radioisotope labelling of metabolic substrates. These tend to be complex, expensive, cumbersome and difficult to standardize. Evaluating the metabolic activity of the intact biofilms in situ as here described obviates many of the technical limitations inherent in other methods. This technique was developed to satisfy the need for a rapid, inexpensive tool capable of meeting the requirement for the

exploding interest in the biology of bacterial biofilms in medicine, with particular reference to the clinical problem of implant-associated infections and CAPD peritonitis.

The method here described for the investigation of bacterial biofilms provides the following advantages:

1) the bacterial morphology, distribution and metabolic activity within the biofilm are examined in their undisturbed state;
2) bypassing the standard technique for superficial viable bacterial counts avoids the necessary crude fragmentation of the biofilm which in turn leads to nonhomogeneity, unpredictable bacterial cell loss and significant errors of enumeration;
3) the undesirable delay between the test exposure of the biofilm to antimicrobial action and the assessment of residual viability is reduced to a minimum;
4) the antimicrobial effect on viability may be effectively measured by simple naked-eye observation;
5) the speed and simplicity of the technique allows the examination of a large number of test variables concurrently;
6) the requirement for specialized equipment, material and training is obviated; and

7) as illustrated in this thesis, both the exposure and the recovery phases can independently be modified for special purposes (Chapter 4.1.6).

S. epidermidis is the commonest cause of peritonitis complicating CAPD. It has been proposed that the resistance to antibiotic therapy of established peritonitis is related to the presence of bacterial biofilms on the permanent peritoneal catheter. This hypothesis would require that bacteria present in biofilms are protected in some manner and do not respond to antibiotics as they do in the fluid phase in standard laboratory testing. We selected a strain of S. epidermidis from human origin known to produce biofilms and known to be fully sensitive in the fluid phase to those antibiotics used in this assay. Results obtained show that the bacterial biofilms did indeed protect the S. epidermidis from the activity of antibiotics.

Rifampin was the sole antibiotic, among 35 tested, to exert a significant antimicrobial effect against S. epidermidis biofilms. Rifampin activity could be observed as early as 6 hours of exposure, whereas none of the other antibiotics had any demonstrable effect even after 24 hours. This outstanding activity of rifampin was maintained in all experiments. Rifampin produced a predominantly bactericidal response with foci of metabolically active residual cells recognized as the phenomenon of resistance. Culture of these viable cells demonstrated unchanged identity and sensitivity profiles with the exception of solid resistance to rifampin both by

standard testing in the fluid phase and in the biofilm phase. To my knowledge this is the first demonstration of this phenomenon in the microenvironment of a biofilm matrix.

The sterilization theory of Watson (222) requires that the concentration of effective agent versus time of exposure has an exponential relationship. Graphically this produces a straight line when a log concentration is plotted against log time. Regarding rifampin activity against biofilm bacteria, figure 12 shows an absence of the expected exponential relationship, indeed graphical analysis of the plot is consistent with a third order polynomial function. The sterilization kinetics of antibiotics is seen to be more complex against biofilm preparations than in the conventional fluid phase.

The mechanism by which antibiotics in general lack effectiveness against bacteria protected by a biofilm is unknown (50). Electrostatic binding or charge repulsion of the antibiotic molecule by the physical organization of the biofilm have been proposed. These data clearly demonstrate that rifampin is able to penetrate the biofilm matrix and gain access to the resident bacteria.

From the clinical standpoint it would be essential for an effective therapeutic regime to eliminate these minor foci of resistance and complete the bacterial killing. A series of experiments were conducted to assess the influence of PD solutions and their additives on their own and in modifying the activity of rifampin on S. epidermidis biofilm preparations. The activity of several antibiotic

combinations incorporating rifampin was evaluated. Of these only the individual combinations of cloxacillin, oxacillin and vancomycin with rifampin were effective in preventing the emergence of rifampin-resistant S. epidermidis, although alone they had no demonstrable activity. The activity of rifampin was also enhanced by falling pH and the presence of fresh PD solutions. Unexpectedly, major antagonism of rifampin activity was demonstrated by spent PD fluids, in the presence of the antibiotics clindamycin, gentamicin, tetracycline and fusidic acid, and by the i.p. drug additives heparin, insulin, calcitriol, urokinase and verapamil.

Clinically relevant to the observation of rifampin activity against bacterial biofilms is the current recommendation for its inclusion in therapeutic regimes for CAPD peritonitis (116) and other infections associated with implanted foreign bodies (5,164,212,221). According to these recommendations, rifampin should be added to vancomycin for the treatment of refractory or relapsing peritonitis caused by Gram positive bacteria. The observed improvement of the therapeutic response by the addition of rifampin is attributed to its action on bacteria present in sequestered sites, either through uptake by phagocytic cells (25) or as the presented data suggest, by penetration into a postulated biofilm matrix directly.

The antimicrobial activity against S. epidermidis biofilms of several closely related formulations of the rifamycin class of antibiotics to which rifampin belongs was

assessed. The chief finding was that the antimicrobial activity against S. epidermidis biofilms is a common property of the members of the rifamycin antibiotic class. However, there were significant intraclass variations. Each of the rifamycin formulations produced demonstrable antimicrobial effects against S. epidermidis biofilms after a few hours of exposure. Conditions of the milieu that are known to enhance the antimicrobial activity of rifampin against bacteria in the fluid phase (low pH, absence of protein), also enhanced activity against the biofilm phase. In the case of spent PD fluid which was markedly antagonistic to the tested formulations, the data indicate that pH played a greater role than protein content. Using different milieus, a number of quantitative and qualitative differences emerged between the various members of the rifamycin class. These data suggest that small changes in two side chains of the basic rifampin molecule are of key importance for antimicrobial activity against S. epidermidis biofilms (Figure 3).

In view of the fact that the two analogs demonstrated antimicrobial activity superior to rifampin, it is tempting to speculate that other analogs might have even greater activity. Because of the morbidity and mortality arising from infections associated with medical implants, many of them related to bacterial biofilms, there is a need to develop new therapeutic strategies for the antibiotic management of these infections incorporating rifampin and to develop new analogs of even greater activity. Furthermore,

these *in vitro* studies clearly demonstrated that the Ciba-Geigy analog (CGP029861) offers the best combination of advantages over the other preparations, including the Merrell-Dow analog (rifapentine), in its ability to withstand a variety of environmental changes likely to occur in the clinical CAPD situation. The superior antimicrobial activity of the Ciba-Geigy analog against *S. epidermidis* biofilm preparations may have therapeutic importance in clinical areas outside of CAPD.

RenNew is a series of stable chlorous acid/chlorine dioxide generating systems of some chemical complexity with a number of formulations designed by the manufacturer for various end purposes and environments (55,121). RenNew-D, a close relative of RenNew-P, has been extensively tested in various systems and combines a pronounced antimicrobial activity with markedly low toxicity and tissue damage in experimental animals. RenNew-P is a formulation directed towards peritoneal dialysis, for both prophylaxis and therapy of CAPD peritonitis. The series of products were evaluated for their activity against *S. epidermidis* biofilms. The four formulations of RenNew-P studied embody variations of the same chlorous acid/chlorine dioxide generating systems.

The time required to sterilize *S. epidermidis* biofilms by RenNewP-1 is similar to that determined for other potent chemical disinfectants. The agents tested included disinfectants of classes used for environmental sterilization (formol saline and hydrogen peroxide) and

other antiseptics specifically utilized in CAPD practice (Amuchina, chlorhexidine, Dakin's solution and povidone-iodine). The wide variation of chemical structure and working strength was reflected in the different times required to sterilize, which varied from 5 seconds to 3 hours.

Dialysis solutions markedly enhanced the activity of RenNewP-1 and the mechanism remains to be explained. The presence of protein had an inhibitory effect on the sterilizing activity of RenNewP-1 in dilution, showing a non-uniform inhibition, a phenomenon which requires further investigation. Similar but more profound inhibition by protein was noted with formol saline, as has indeed been demonstrated for numerous classes of disinfectants. Diluting RenNewP-1 resulted in an exponential increase in sterilizing time as with formol saline. This parallels observations on dilution/time relationships of many disinfectants acting against bacteria in the fluid phase (30,222). These results with chemical agents are identical for the fluid phase of S. epidermidis. This contrasts greatly with observations on antibiotic activity against the same bacteria in the biofilm phase, suggesting that a complex interaction develops between antibiotic molecules and the biofilm matrix.

The relevance of this data to clinical practice lies in the likelihood of disinfecting agents interacting with peritoneal dialysis solutions, by accident or design, or with protein-containing spent dialysis fluid, particularly

during episodes of peritonitis (61). Further investigations were undertaken to verify whether spent dialysis fluids would inhibit disinfectant activity against S. epidermidis biofilms as was demonstrated for rifampin both alone and in otherwise effective antibiotic combinations. The deliberate addition of chemical disinfecting agents to commercial peritoneal dialysis solutions has been proposed for routine prophylaxis. The instillation of these additives in the peritoneal cavity as a flush or a lavage in the treatment of established CAPD peritonitis has already been addressed and remains speculative (65). RenNew-P, which demonstrates marked antimicrobial activity against S. epidermidis biofilms, if coupled with the anticipated low tissue toxicity, indicates a significant role for this class of compounds in the prophylaxis of CAPD peritonitis.

The clinical relevance of bacterial biofilms associated with the peritoneal catheters of CAPD patients remains to be defined. Dasgupta et al (51,53) have demonstrated that the amount and distribution pattern of the bacterial biofilms on the catheter surfaces were comparable in all CAPD patients. Biofilms were present on the surface of all recovered CAPD peritoneal catheters, irrespective of whether the patients had peritonitis or other catheter-related infections such as tunnel and exit site infections or whether they had been infection-free for at least 3 months (51,53,145,184). It can be argued that the universality of these findings excludes a role for S. epidermidis biofilms in the pathogenesis of catheter-associated sepsis. However, this

argument ignores the possibility that biofilms may exist in states of differing metabolic activity and pathogenic potential at different times. Furthermore, possible alterations may exist in local immune reactivity to the biofilms secondary to the uremic state and the i.p. presence of PD solutions and therapeutic drug additives. I have demonstrated in this work a significant cytotoxicity and functional impairment of mouse peritoneal cells induced primarily by PD solutions.

Currently, the pathogenesis of CAPD peritonitis remains unclear. Bacterial biofilms may play a number of roles in the development of sepsis including the direct seeding in the peritoneal cavity and the impairment of host defense mechanisms. Recent observations suggest that the slime-like substance of bacterial biofilms purified from S. aureus and S. epidermidis are potent inhibitors of most phagocytic cell functions (89,90,113,159). If biofilms have the capability of varying states of pathogenic potential, these variations can be the subject of separate studies. Current studies are severely hampered by the lack of relevant animal models; these are warranted to determine if the in vitro findings as well as the significant, albeit limited, in vivo results (superior efficacy of rifampin) demonstrated here can be duplicated and confirmed elsewhere.

It was shown, as others have done with different systems, that bacterial biofilms exhibit resistance to the action of many antibiotics. The mechanisms underlying the resistance of bacterial biofilms to antibiotics remain

unknown. An explanation suggested is the physical ordering of the polymer structure of the glycocalyx matrix donating biologically advantageous properties. The development of resistance of fluid phase bacteria to antibiotic action is known to be the result of one or more of three phenomena: 1) extracellular inactivation of antibiotics by enzymatic digestion, 2) loss of cell membrane receptors, and 3) alteration in the target site within the bacterial cell. In the case of biofilm phase bacteria, it is apparent that the demonstrated resistance implies yet a fourth mechanism reducing antibiotic access. Clearly this mechanism is a function of the properties of the intact biofilm itself. Such a mechanism has not yet been described.

As previously indicated, rifampin was the only effective antibiotic against S. epidermidis biofilms among 35 antibiotics tested. This antibiotic is recognized to have unique pharmacokinetic properties which have broadened the spectrum of its clinical use to nontuberculous infections (193,210). How these properties render rifampin active against S. epidermidis biofilms is speculative. Rifampin is highly lipid soluble, a property that allows it to penetrate into various restricted sites including abscess cavities and crossing the blood brain barrier into the central nervous system (69). It is one of few antimicrobial agents that is capable of killing bacteria sequestered within the protective environment of phagocytic cells (138,180). Clindamycin which is also lipid soluble and which also demonstrated intracellular penetration and

killing (122,138) is, in contrast to rifampin, ineffective in the studies against S. epidermidis biofilms, an unexpected finding. This indicates a fundamental difference in relevant properties. Recent experimental and clinical studies of rifampin therapy in foreign body infections confirm the validity of the in vitro data by demonstrating empirically its value (5,72,116,212). One can speculate that the rifampin molecule, perhaps by the nature of electrical charge, is able to overcome the impairment of penetration that may be exerted by the intact glycocalyx structure. One property of rifampin shared by the majority of amino acids is the presence of zwitterions, altering the charge of the molecule.

I conclude that the present standardized biofilm test system is a valuable technique for the evaluation of antimicrobial activity alone or in combination with different milieus. It is recognized that there is a wide variation and clinical unpredictability of outcome in prosthesis associated infections. I have demonstrated similar wide variation of behavior for bacteria in the biofilm phase contrasted to the fluid phase suggesting the desirability of further studies of these phenomena. The mouse model of catheter/biofilm infection will aid in exploring host defense mechanisms to bacterial biofilms and in investigating possible therapeutic regimes, especially including those antimicrobials shown to be effective in the in vitro biofilm assay.

CHAPTER 6

FUTURE DIRECTIONS

The research endeavoured for this thesis has demonstrated the marked significant difference in the activity of antimicrobials directed against bacteria in the fluid phase and in the biofilm phase. Although a direct causal relationship between bacterial biofilms and infection remains elusive, foreign implanted medical devices including CAPD catheters are known to be repositories for infectious viable pathogens. Extrapolating the findings of these investigations to the clinical situation during episodes of persisting or recurrent S. epidermidis peritonitis in CAPD could possibly implicate biofilms with infection. The clear importance of introducing a method, as here described, for standard routine microbiological testing to aid in the diagnosis of clinical infections associated with foreign implanted bodies is most evident.

In analysing the results and the potential of this novel method for biofilm investigation, numerous future directions remain to be addressed. These may involve the mechanisms behind the superior efficacy of rifampin and analogs against S. epidermidis biofilms as well as a delineation of the structural moieties responsible for differences in activity, including studies of molecular charge characteristics. Further elucidation of the activity

of each class of antimicrobial agents tested in various exposure media including the striking antagonistic properties of spent peritoneal dialysis fluid is also warranted.

This thesis investigated minimal density S. epidermidis biofilm preparations. Additional experiments are necessary to assess the antimicrobial activity of rifampin and analogs against more developed S. epidermidis biofilms. Furthermore, the use of this method or a modification thereof to investigate other biofilm forming microorganisms in vitro and similarly assess the activity of a more extensive antibiotic screen is also warranted.

The albeit limited investigation of S. epidermidis biofilms in the mouse model demonstrated the inability of the host defenses of normal animals to spontaneously eradicate the bacterial biofilm. Furthermore and most relevant to the thesis, I was able to confirm in the mouse model of biofilm infection the superior antimicrobial activity of rifampin which was observed in vitro. Clearly more in depth animal studies are required for an extensive assessment of host defenses, antibiotherapy and uremia on catheter adherent bacterial biofilms.

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APPENDIX 1

Characteristics of the Clinical Isolate, *Staphylococcus epidermidis*

A clinical isolate of *S. epidermidis* was recovered from a patient with CAPD peritonitis, subcultured to ensure purity and thereafter frozen in aliquots for use in all experiments described in this thesis. The characteristic appearance of this particular strain grown on standard blood agar were of white, glossy, circular colonies (1 mm in diameter following 18 hours incubation at 37°C). In addition to standard assessments by Gram stain (positive), catalase (positive) and coagulase (negative) production this strain also underwent analysis in the Vitek test system (McDonnell Douglas Health Systems Company, MO, USA) for antimicrobial susceptiblity and nutrient substrate utilization. These results are presented in Tables 1 and 2, respectively. Other results obtained by the Vitek process indicated a high degree of tolerance to bile and sodium chloride, and reactivity with hemicellulase. This bacteria was further identified as a prolific producer of slime in accordance with a tube test assay (35). Most significant to the research presented here was the reduction of TTC by this strain of *S. epidermidis*. All viable bacteria which incorporated TTC and formed the insoluble red formazan precipitate remained fully viable on subsequent subcultures.

Table 1. Vitek test system: analysis of antimicrobial susceptibility of fluid phase Staphylococcus epidermidis.

	Antibiotics
Sensitivity:	ampicillin, cephalothin, chloramphenicol, erythromycin, gentamicin, nitrofurantoin, penicillin, tetracycline, vancomycin, oxacillin
Resistance:	not demonstrated

Table 2. Vitek test system: analysis of nutrient substrate utilization of fluid phase Staphylococcus epidermidis.

Nutrient Substrate	Utilization
Arabinose	-
Cellobiose	-
Dextrose	+
Inulin	-
Lactose	+
Mannitol	-
Melibiose	-
Melezitose	-
Pullulan	-
Pyruvate	-
Raffinose	-
Ribose	-
Salicin	-
Sorbitol	-
Sucrose	+
Trehalose	-
Xylose	-

APPENDIX 2

In vitro Assessment of *Staphylococcus epidermidis* Biofilm/Catheter-Complex Preparations

This Appendix consists of a series of nine colour plates illustrating the naked eye appearance of peritoneal catheter segments with adherent *S. epidermidis* biofilms having been exposed to various *in vitro* conditions. These studies were part of preliminary experiments to the *in vivo* investigations described in Chapter 4.2.

In the first two plates bacterial biofilms at two stages of development (1-day and 3-day growth) are demonstrated by three staining techniques: Gram stain to identify bacterial cells, TTC to detect bacterial viability, and toluidine blue to reveal the presence of the polysaccharide biofilm matrix.

The following three plates show the influence of an 18-hour exposure to different milieus (peptone water and fresh 1.5% PD solution) on the growth of bacterial biofilms at various stages of development (1-day and 3-day growth) as assessed by Gram stain, TTC and toluidine blue.

The last four plates illustrate the response of bacterial biofilms at various stages of development (1-day and 3-day growth) to an 18-hour antibiotic treatment in different milieus (peptone water and fresh 1.5% PD solution). The antimicrobial agents consisted of rifampin, vancomycin and the combination, each antibiotic at a

concentration of 10 µg/ml.

STAPHYLOCOCCUS EPIDERMIDIS BIOFILMS

(1 DAY GROWTH)

GRAM STAIN

TOLUIDINE BLUE

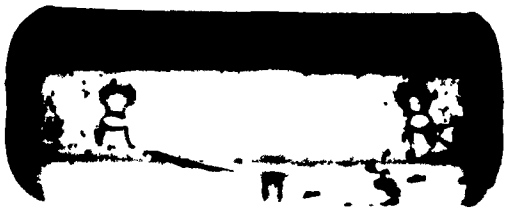
TTC SOLUTION

STAPHYLOCOCCUS EPIDERMIDIS BIOFILMS

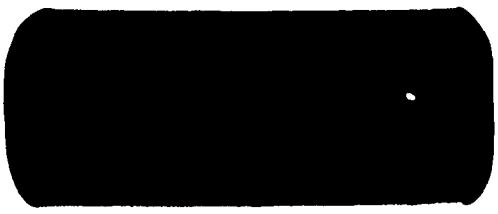
(3 DAY GROWTH)

GRAM STAIN

TOLUIDINE BLUE



TTC SOLUTION



STAPHYLOCOCCUS EPIDERMIDIS BIOFILMS

(GRAM STAIN)

---

1 DAY GROWTH

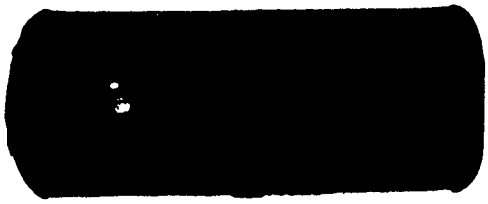
PEPTONE WATER

PD SOLUTION

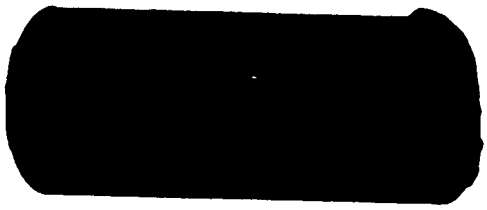
---

3 DAY GROWTH

PEPTONE WATER



PD SOLUTION



STAPHYLOCOCCUS EPIDERMIDIS BIOFILMS

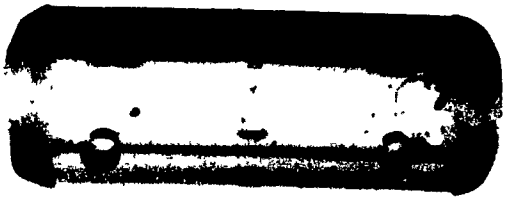
(TTC SOLUTION)

---

1 DAY GROWTH

PEPTONE WATER

PD SOLUTION



---

3 DAY GROWTH

PEPTONE WATER

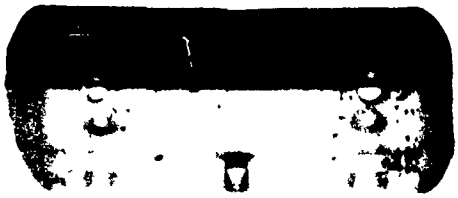
PD SOLUTION

STAPHYLOCOCCUS EPIDERMIDIS BIOFILMS

(TOLUIDINE BLUE)

1 DAY GROWTH

PEPTONE WATER



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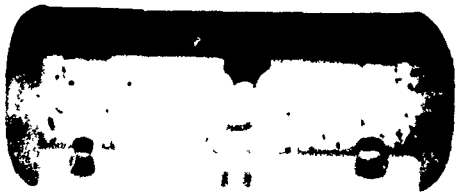
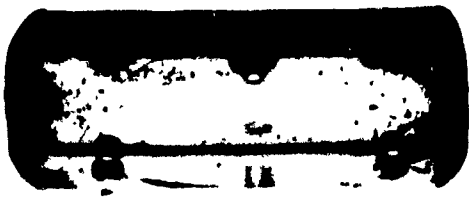


3 DAY GROWTH

PEPTONE WATER



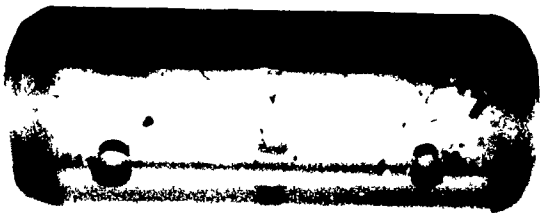
PD SOLUTION

STAPHYLOCOCCUS EPIDERMIDIS BIOFILMS

(1 DAY GROWTH)

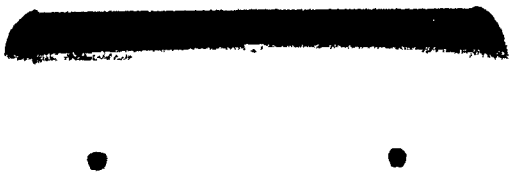
EXPOSED TO:

PEPTONE WATER CONTROL

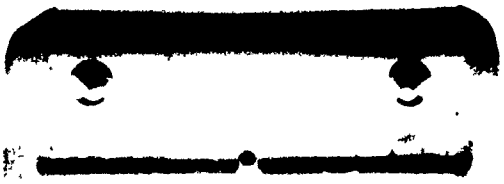


RIFAMPIN (10 µg/ml)

VANCOMYCIN (10 µg/ml)



RIFAMPIN (10 µg/ml) AND VANCOMYCIN (10 µg/ml)



STAPHYLOCOCCUS EPIDERMIDIS BIOFILMS

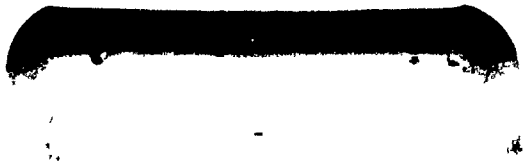
(1 DAY GROWTH)

EXPOSED TO:

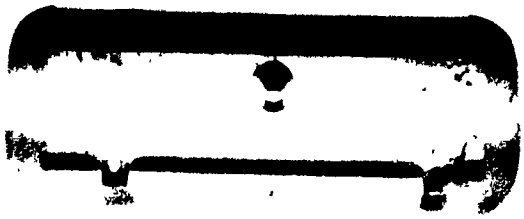
PD SOLUTION CONTROL

RIFAMPIN (10 µg/ml)

VANCOMYCIN (10 µg/ml)



RIFAMPIN (10 µg/ml) AND VANCOMYCIN (10 µg/ml)



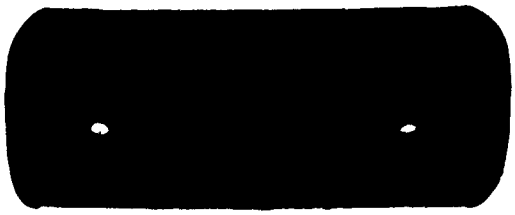
STAPHYLOCOCCUS EPIDERMIDIS BIOFILMS

(3 DAY GROWTH)

EXPOSED TO:

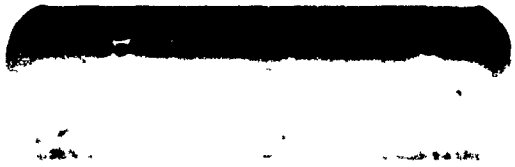
PEPTONE WATER

CONTROL



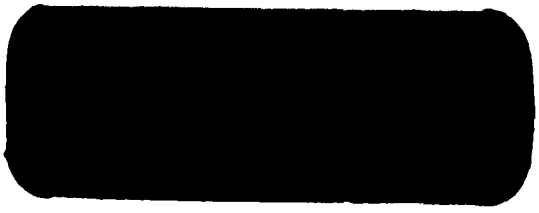
RIFAMPIN

(10 µg/ml)



VANCOMYCIN

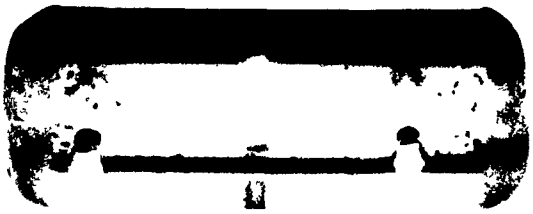
(10 µg/ml)



RIFAMPIN (10 µg/ml)

AND

VANCOMYCIN (10 µg/ml)



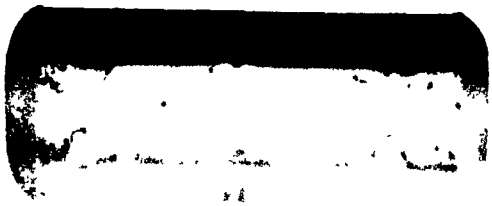
STAPHYLOCOCCUS EPIDERMIDIS BIOFILMS

(3 DAY GROWTH)

EXPOSED TO:

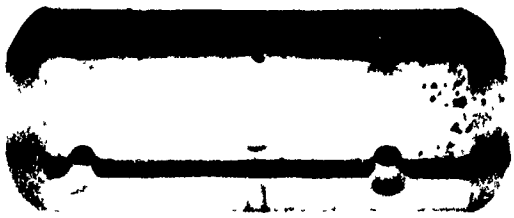
PD SOLUTION
CONTROL

RIFAMPIN
(10 µg/ml)



VANCOMYCIN
(10 µg/ml)

RIFAMPIN (10 µg/ml)
AND
VANCOMYCIN (10 µg/ml)



APPENDIX 3

List of Publications

A. PAPERS

1. Obst G, Gagnon RF, Prentis J and Richards GK. Comparative study of antibiotic activity against Staphylococcus epidermidis biofilms. Proceedings of the 8th National CAPD Conference, 1988.

2. Obst G, Gagnon RF, Prentis J and Richards GK. Sterilization of Staphylococcus epidermidis biofilms by RenNew-P and common disinfectants. Proceedings of the 8th National CAPD Conference, 1988.

3. Obst G, Gagnon RF, Prentis J and Richards GK. Rifampin activity against Staphylococcus epidermidis biofilms. Transactions of the 34th Annual ASAIO Meeting, 1988.

4. Richards GK, Obst G, Prentis J and Gagnon RF. A standardized technique to assess the viability of bacterial biofilms. Submitted to Clin Invest Med.

5. Obst G, Gagnon RF, Prentis J and Richards GK. Effects of peritoneal dialysis solutions and additives on antibiotic activity against Staphylococcus epidermidis biofilms. Submitted to Kidney Int.

6. Obst G, Gagnon RF, Harris A, Prentis J and Richards GK. The activity of rifampin and analogs against Staphylococcus epidermidis biofilms in a CAPD environment model. Submitted to Am J Nephrol.

7. Obst G, Gagnon RF and Richards GK. Peritoneal catheter-associated Staphylococcus epidermidis biofilm preparation in the mouse: response to rifampin. Submitted to Am J Kidney Dis.

8. Obst G, Gagnon RF, Prentis J and Richards GK. The effect of biofilm and milieu on the response of Staphylococcus epidermidis to antibiotics. Submitted to Clin Invest Med.

9. Obst G, Gagnon RF, Richards GK and Gervais F. Effect of drug additives to peritoneal dialysis solutions on the viability and function of mouse peritoneal cells. Submitted to Nephron.

B. ABSTRACTS

1. Obst G, Gagnon RF, Prentis J and Richards GK. A new method of *in vitro* bacterial biofilm formation and assessment of its growth. Conference on Peritoneum and Peritoneal Access, Lund, Sweden, June 22-26, 1987.

2. Obst G and Gagnon RF. *In vitro* assessment of additives to peritoneal dialysis solutions in a cytotoxicity assay against mouse peritoneal cells. Conference on Peritoneum and Peritoneal Access, Lund, Sweden, June 22-26, 1987.

3. Obst G and Gagnon RF. Cytotoxicité de médicaments administrés par voie péritonéale envers des cellules péritonéales de souris. 29th Annual Meeting of the Club de Recherches Cliniques du Québec, Montebello, Canada, October 29-31, 1987.

4. Richards GK, Obst G, Gagnon RF and Prentis J. A new bacterial biofilm assay. 8th National CAPD Conference, Kansas City, Missouri, USA, February 10-12, 1988.

5. Obst G, Gagnon RF, Prentis J and Richards GK. Comparative study of antibiotics in a bacterial biofilm assay. 8th National CAPD Conference, Kansas City, Missouri, USA, February 10-12, 1988.

6. Obst G, Gagnon RF, Prentis J and Richards GK. Evaluation of a new antiseptic, RenNew-P, in a bacterial biofilm assay. 8th National CAPD Conference, Kansas City, Missouri, USA, February 10-12, 1988.

7. Richards GK, Obst G, Prentis J and Gagnon RF. A rapid method for assessment of antimicrobials against bacterial biofilms. 34th Annual ASAIO Meeting, Reno, Nevada, USA, May 3-6, 1987.

8. Obst G, Gagnon RF, Prentis J and Richards GK. Effects of peritoneal dialysis solutions on the activity of antimicrobial agents against *S. epidermidis* biofilms. 34th Annual ASAIO Meeting, Reno, Nevada, USA, May 3-6, 1987.

9. Obst G, Gagnon RF, Prentis J and Richards GK. Rifampin activity against *S. epidermidis* biofilms. 34th Annual ASAIO Meeting, Reno, Nevada, USA, May 3-6, 1987.

10. Richards GK, Obst G, Prentis J and Gagnon RF. Metabolic indicators for the investigation of intact bacterial biofilms. 57th Annual Meeting of the Royal College of Physicians and Surgeons of Canada, Ottawa, Canada, September 23-26, 1988.

11. Obst G, Gagnon RF, Prentis J and Richards GK. Metabolic evaluation of the antimicrobial activity of rifampin against Staphylococcus epidermidis biofilms. 57th Annual Meeting of the Royal College of Physicians and Surgeons of Canada, Ottawa, Canada, September 23-26, 1988.

12. Obst G, Gagnon RF, Prentis J and Richards GK. Comparison of fresh and spent peritoneal dialysis solutions on the activity of antimicrobial agents against Staphylococcus epidermidis biofilms. 57th Annual Meeting of the Royal College of Physicians and Surgeons of Canada, Ottawa, Canada, September 23-26, 1988.

13. Richards GK, Obst G, Prentis J and Gagnon RF. Mise au point d'une technique d'investigation des biofilms bactériens. 30th Annual Meeting of the Club de Recherches Cliniques du Québec, Pointe-au-Pic, Canada, October 6-8, 1988.

14. Obst G, Gagnon RF, Prentis J and Richards GK. Activité de la rifampine envers des biofilms de Staphylococcus epidermidis. 30th Annual Meeting of the Club de Recherches Cliniques du Québec, Pointe-au-Pic, Canada, October 6-8, 1988.

15. Gagnon RF, Obst G, Prentis J and Richards GK. Effect of peritoneal dialysis solutions on the activity of antibiotic and disinfecting agents against Staphylococcus epidermidis biofilms. 21st Annual Meeting of the American Society of Nephrology, San Antonio, Texas, USA, December 11-14, 1988.

16. Harris A, Obst G, Gagnon RF, Prentis J and Richards GK. Antimicrobial activity of rifamycin antibiotics against Staphylococcus epidermidis biofilms in a CAPD environment model. 21st Annual Meeting of the American Society of Nephrology, San Antonio, Texas, USA, December 11-14, 1988.

17. Obst G, Gagnon RF, Prentis J and Richards GK. Modulation of antibiotic activity against Staphylococcus epidermidis biofilms by therapeutic drugs added to peritoneal dialysis (PD) solutions. 21st Annual Meeting of the American Society of Nephrology, San Antonio, Texas, USA, December 11-14, 1988.

18. Richards GK, Obst G, Prentis J and Gagnon RF. A recovery indicator method for the investigation of intact bacterial biofilms. 21st Annual Meeting of the American Society of Nephrology, San Antonio, Texas, USA, December 11-14, 1988.