Antibiotic action against S. epidermidis biofilms
Modulation by CAPD variables

Antibiotic action against experimental *Staphylococcus epidermidis* biofilms as an *in vitro* analogue of catheter-associated infection in continuous ambulatory peritoneal dialysis: Modulation by CAPD variables

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For My Parents and Grandfather,...

ABSTRACT

Continuous ambulatory peritoneal dialysis (CAPD) is a form of renal replacement therapy for patients with end-stage renal disease. Peritonitis and peritoneal catheter-associated infections are major complications of CAPD and may lead to the abandonment of this valuable mode of therapy. These infections are predominantly due to Staphylococcus epidermidis, a skin commensal of limited pathogenicity in the absence of implanted material, and are characterized by an indolent course (evidence of resistance to host defenses) and poor responses to antibiotic therapy. This resistance has been ascribed to the property of S. epidermidis to adhere readily to artificial surfaces and produce a protective coating of a polysaccharide matrix to form a bacterial biofilm, exerting a selective or absolute impermeability to harmful agents.

The properties of the biofilm matrix were studied in a standardized in vitro analogue of a catheter-bacterial biofilm infection, to determine the nature of the selective permeability. The biofilm environment was modified by CAPD variables to see if the biofilm permeability could be altered, using the kinetics of antibiotic action against the enclosed bacteria as a measure of change.

Amongst the antibiotics examined, rifampin was found to be the most rapid in action, depleting the bacterial content within hours to a few scanty resistant survivors which then progressively repopulated the biofilm. Other antibiotics were considerably slower in penetrating the biofilm, a bactericidal outcome being measured in days. The well-studied sequence of rapid but incomplete bactericidal action by rifampin was used as a kinetic measure to determine the modulating effect of modifications in the biofilm environment on this selective permeability.

The environment was modified by the addition of antibiotics of clinical relevance in various combinations, by exposure to peritoneal dialysis (PD) solutions of various formulations, by pooled spent fluid, and by non-antibiotic therapeutic agents commonly added to PD solutions.

The study was successful and showed that the permeability of the biofilm could be significantly altered. Unexpected synergistic and antagonistic interactions were found, particularly with antibiotic combinations. Fresh PD solutions of all formulations were synergistic, completing the bactericidal effect of rifampin. Spent fluid demonstrated significantly lesser synergy. Therapeutic non-antibiotic additives showed no alterations of biofilm permeability, with the interesting exception of urokinase.

The studies confirmed the shielding properties of the bacterial biofilm and its selective permeability to therapeutic agents. It was determined that the permeability could be altered significantly, both adversely and favorably, by modification of the biofilm environment with CAPD variables. The study indicated the potential for clinical application of these findings and the potential to determine logically optimization of therapeutic regimes for the management of medical device infections.

RÉSUMÉ

La dialyse péritonéale ambulatoire continue (DPAC) est une forme de thérapie de remplacement rénal pour les malades avec insuffisance rénale terminale. La péritonite et les infections associées au cathéter péritonéal sont des complications majeures de la DPAC et peuvent mener à l'abandon de ce mode important de traitement. Ces infections sont dues d'une manière prédominante au Staphylococcus epidermidis, une bactérie commensale de pathogénicité limitée en l'absence de matérial implanté, et sont caractérisées par un cours indolent (évidence de résistance aux mécanismes de défenses de l'hôte) et des réponses faibles à l'antibiothérapie. Cette résistance a été attribuée à la propriété du S. epidermidis d'adhérer facilement aux surfaces artificielles et de produire un revêtement protecteur d'une matrice de polysaccharide pour former un biofilm bactérien, exerçant une imperméabilité sélective ou absolue pour les agents nocifs.

Les propriétés de la matrice du biofilm ont été étudiées dans un analogue standardisé in vitro d'une infection de cathéter avec biofilm bactérien, afin de déterminer la nature de la perméabilité sélective. L'environnement du biofilm a été modifié par des variables de la DPAC pour voir si la perméabilité du biofilm pouvait être altérée, utilisant la cinétique de l'action antibiotique contre les bactéries incluses comme mesure de changement.

Parmi les antibiotiques examinés, la rifampicine a été trouvée la plus rapide d'action, épuisant le contenu bactérien en quelques heures à de rares survivants résistants qui par la suite repeuplèrent progressivement le biofilm. D'autres antibiotiques ont été considérablement plus lents à pénétrer le biofilm, un résultat bactéricide étant mesuré en jours. La séquence bien étudiée de l'action bactéricide rapide mais incomplète par la rifampicine a été utilisée comme mesure cinétique pour déterminer l'effet modulateur de changements de l'environnement du biofilm sur la perméabilité sélective.

L'environnement a été modifié par l'addition d'antibiotiques de pertinence clinique en combinaisons diverses, par l'exposition à des solutions de dialyse péritonéale (DP) de formulations variées, à du liquide de drainage péritonéal groupé, et à des agents thérapeutiques non-antibiotiques fréquemment ajoutés aux solutions de DP.

L'étude a été réussie et a montré que la perméabilité du biofilm pouvait être altérée d'une manière significative. Des interactions inattendues synergiques et antagoniques ont été trouvées, particulièrement avec les combinaisons antibiotiques. Les solutions fraiches de DP de toutes formulations ont été synergiques, complétant l'effet bactéricide de la rifampicine. Le liquide de drainage a démontré une synergie beaucoup moindre. Les ajouts thérapeutiques non-antibiotiques ont montré aucunes altérations de la perméabilité du biofilm, avec l'exception intéressante de l'urokinase.

Ces études ont confirmé les propriétés protectrices du biofilm bactérien et la perméabilité sélective aux agents thérapeutiques. Il a été déterminé que la perméabilité pouvait être altérée de manière significative, autant de façon adverse que favorable, par la modification de l'environnement du biofilm par les variables de la DPAC. Cette étude a indiqué le potentiel pour l'application clinique de ces observations et le potentiel de déterminer logiquement l'optimisation des régimes thérapeutiques pour le traitement des infections reliées aux implants médicaux.

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CAPD	Continuous ambulatory peritoneal dialysis
cfu	Colony forming unit
C-NS	Coagulase-negative staphylococci
°C	Degree centigrade
CORR	Canadian Organ Replacement Register
DNA	Deoxyribonucleic acid
EPS	Exopolysaccharide substance
ESRD	End-stage renal disease
FITC	Fluorescein isothiocyanate
g	Gram
Hep	Heparin
hr	Hour
Ins	Insulin
i.p.	Intraperitoneal
kg	Kilogram
L	Liter
mg	Milligram
MGH	Montreal General Hospital
MH	Mueller Hinton
min	Minute
MIC	Minimal inhibitory concentration
ml	Milliliter
mm	Millimeter
mmol	Millimolar
mOsm/kg H_2O	Milliosmole per kilogram of water
µg	Microgram
µl	Microliter
µm	Micrometer
MW	Molecular weight
NaOH	Sodium hydroxide
NCCLS	National Committee for Clinical Laboratory Standards
NK	Natural killer cells
nm	Nanometer

P. aeruginosa	Pseudomonas aeruginosa
P. fluorescens	Pseudomonas fluorescens
PD	Peritoneal dialysis
PGE	Prostaglandin E
pH	Potenz hydrogen
PMN	Polymorphonuclear leukocytes
PTFE	Polytetrafluoroethylene
PW	Peptone water
RNA	Ribonucleic acid
RR	Rifampin-resistant
RS	Rifampin-sensitive
S. aureus	Staphylococcus aureus
S. epidermidis	Staphylococcus epidermidis
SCLM	Scanning confocal laser microscope
SEM	Scanning electron microscopy
sec	Second
T-cell	Thymus-derived lymphocyte cells
TEM	Transmission electron microscopy
TSB	Tryptone soya broth
TTC	2,3,5-triphenyltetrazolium chloride
U	Unit
Uro	Urokinase

HYPOTHESIS

In patients with end-stage renal disease on continuous ambulatory peritoneal dialysis (CAPD), the resistance of peritonitis caused by Staphylococcus epidermidis to conventional antibiotic therapy is due to the presence of the bacteria in the biofilm phase on the surface of the permanent peritoneal catheter. The biofilm matrix provides protection to the enclosed bacteria.

The intraperitoneal environment of CAPD (peritoneal dialysis solutions and non-antibiotic therapeutic additives) can modulate the antimicrobial activity of antibiotics against S. epidermidis in the biofilm phase.

Using an in vitro analogue of catheter-associated infection (standardized S. epidermidis biofilms formed on an artificial surface), the interaction between the microbe and antibiotics in the presence of varying intraperitoneal environments can be studied.

INTRODUCTION

Chronic renal failure refers to the slowly progressive reduction of renal function. Irrespective of the cause of the renal failure, the chronic retention of substances normally excreted by the kidneys results in profound complex metabolic derangements affecting the function of all main physiological body systems. Chronic renal failure can progress to reach a stage where patients require renal replacement therapy by dialysis or kidney transplantation in order to survive.

1. Continuous Ambulatory Peritoneal Dialysis

Continuous ambulatory peritoneal dialysis (CAPD) is a form of renal replacement therapy which was introduced in 1976 for patients with end-stage renal disease (ESRD) (43, 109). This technique has received widespread acceptance worldwide and its use has markedly increased over the past several years. In Canada the number of CAPD patients has doubled over the past 10 years (Figure 1).

CAPD demonstrates many advantages over hemodialysis and in some patients is the preferred choice for renal replacement therapy. In patients undergoing CAPD, dietary intakes of protein, sodium, potassium and fluids can usually be more liberal than in patients on hemodialysis. CAPD can provide nearly steady-state blood chemistry and blood pressure control in patients with cardiovascular disease which is not demonstrated in hemodialysis. CAPD does not induce the symptoms of disequilibrium which is common with hemodialysis and does not require access to the circulation and anticoagulation (102).

Ganter, a German clinical investigator, is credited with the first attempts of peritoneal dialysis in 1918 in humans by injecting saline into the peritoneal cavity and observing almost complete equilibration of the non-protein nitrogen content in the patient blood and the peritoneal cavity following a dwell time of 3 hours (52). Several years later Ganter's technique was modified by inserting two catheters in the peritoneal cavity, where one catheter was located between the diaphragm and the liver for the inflow of dialysis fluid while the other catheter was placed in the pelvic area for the outflow of dialysis fluid. However, much of the attempts to approach renal clearance were unsuccessful (39).

Many years followed with futile attempts at the chemical epuration of blood by dialysis until 1946 when Frank and colleagues reported the successful application of peritoneal lavage in a patient with severe renal failure from sulphathiozol-induced anuria (44). This experimental treatment resulted in the survival of the patient. Further applications of this treatment in patients with acute renal failure of various etiology were also successful.

The successful application of intermittent peritoneal dialysis in patients with acute renal failure led to the investigation of repeated peritoneal dialysis in a patient with chronic renal failure (141). An initial dialysis treatment significantly improved the physiological state of the patient such that another consecutive treatment was performed. With continued success in this patient, "periodic" peritoneal dialysis followed. The patient survived for one year as a result of the treatment.

In 1976 Popovich and colleagues described a novel portable equilibrium peritoneal dialysis (PD) technique based on the rationale that acceptable blood metabolite levels will result if the dialysis fluid is allowed to continuously equilibrate with body fluids (122). This concept suggested that the continuous presence of PD fluid in the peritoneal cavity, interrupted by periods for drainage

and instillation of fresh dialysis solution approximately 5 times/day, could represent a viable form of renal replacement therapy for ESRD patients. After extensive clinical studies, this therapy was introduced under the name of CAPD for continuous ambulatory peritoneal dialysis (123).

CAPD has now gained widespread acceptance as an alternative to hemodialysis for ESRD patients. This technique is simple, can be handled by the patient alone, and is therefore cost-efficient. Solute exchange between the peritoneal microcirculation and peritoneal cavity occurs by diffusion. In CAPD the relative insufficiency of peritoneal dialysis for the removal of the metabolic byproducts present in renal failure is mostly compensated by the fact that the process is continuous over 24 hours 7 days a week (79, 82, 123).

Comparisons of clearances of molecules of various sizes by CAPD and hemodialysis have demonstrated the superiority of CAPD in clearing larger molecules. Weekly values for the clearance of urea (MW: 60) are lower in CAPD than hemodialysis performed 3 times a week. However weekly values for the clearance of inulin (MW: 5000) are higher in CAPD compared to hemodialysis where very little inulin is cleared (103). Babb noticed that the removal of large molecules, so-called "middle molecules" (MW: 300-2000) is more efficient in CAPD than hemodialysis while the clearance of small molecules were only 1/6 to 1/4 of those with hemodialyzers (5). Higher molecular weight solutes (e.g. inulin) were removed at higher rates than urea and other small molecules compared with hemodialysis. The longer peritoneal dialysis time allowed for the reduction of small molecule concentrations to acceptable levels.

Fluid removal in CAPD is achieved by ultrafiltration using osmotic forces. PD solutions contain electrolytes (e.g. Na^+, K^+, Cl^-), a buffer (usually lactate) and dextrose, currently the most commonly used osmotic agent. The osmotic

agent of the PD solution that is infused into the peritoneal cavity, creates a high osmotic pressure inducing water flux primarily from the peritoneal capillaries into the peritoneal cavity. When 2 liters of dialysis solutions are introduced into the peritoneal cavity during a cycle, the transcapillary forces that are normally present are altered in favour of enhanced ultrafiltration. Maximal ultrafiltration occurs at the beginning of the exchange period and decreases as the dextrose concentration is dissipated via the combining effect of the dilution of the ultrafiltrate leading to decreased levels of osmotic pressure and from the absorption of the osmotic agent into the circulation by the lymphatics or directly by the peritoneal capillaries. However, the peritoneal lymphatics continuously absorb intraperitoneal fluid by convective flow whereas the absorption by the peritoneal capillaries is non continuous due to its dependence on the balance of hydrostatic pressure and the osmotic transperitoneal pressure gradients (58, 92).

2. CAPD Catheter-related Infections

Infections related with the indwelling peritoneal catheter are commonly observed in CAPD patients and represent the main limitation of this otherwise successful form of renal replacement therapy. The infections occur at any location related to the catheter whether at the skin exit site (exit site infection), in the tissue surrounding the catheter all along its tract from the skin to the peritoneal cavity (tunnel infection) and in the peritoneal cavity itself (peritonitis). The latter infection represents the main medical reason for the discontinuation of CAPD (Figure 2). Peritonitis is also a major contributing factor to several other identifiable reasons for the discontinuation of CAPD therapy e.g. patient unable to cope and inadequate dialysis (3).

Among the many underlying factors involved in CAPD infections, the most important factor is that the catheter is permanently exposed to the exterior through the skin while the other end is situated in the peritoneal cavity allowing for the propagation of a variety of microorganisms from the skin surface inwardly towards the peritoneal cavity (11, 21, 30).

Exit Site and Tunnel Infections

Exit site and CAPD tunnel infections are frequent and are major causes of morbidity and of removal of the peritoneal catheter with discontinuation of CAPD. When infected, the exit site is erythematous and elevated, draining pus or serous fluid but usually not painful (25). Staphylococcus aureus is the most common cause of exit site and tunnel infections. S. aureus and Pseudomonas are the main causative organisms leading to catheter removal due to exit site infection.

These infections have the potential of causing peritonitis via migration of bacteria into the peritoneal cavity through the periluminal route (120). Extensive clinical studies have shown that approximately 20% of all peritonitis episodes recur as a result of exit site or tunnel infections (120). The risk of peritonitis is considered to double following an exit site infection.

The most significant recent advance in the prevention of exit site and tunnel infections has been the optimization of the exit site care during the immediate post-operative period after implantation of the catheter enabling better tissue fixation of the catheter and tighter seal at the skin exit site. Further improvement has been related to the strict adherence to sterile technique in chronic catheter care (25).

Peritonitis

Since the introduction of CAPD, peritonitis remains the most significant complication of this form of renal replacement therapy. CAPD peritonitis is

clearly distinguishable from surgical peritonitis in several important aspects (Table 1). First, peritoneal infection is a rare complication in abdominal surgery, developing only after gross contamination of the peritoneal cavity by intestinal content (65). In contrast, CAPD peritonitis is frequent usually occurring following minor episodes of touch contamination (151). As a consequence, the pathogenic organisms causing peritonitis are different in those two clinical situations. Surgical peritonitis is due to enteric organisms whereas CAPD peritonitis is due mainly to indigenous skin organisms. Because of the permanent presence of PD solutions, infections diffuse readily within the peritoneal cavity; however, toxic manifestations and fever are often lacking in CAPD patients and early signs and symptoms are difficult to identify by the patient because of the periodic drainage of inflammatory reactants (137). Moreover, blood cultures are constantly negative during CAPD peritonitis, whereas evidence for bacteremia is demonstrated in a significant percentage of surgical peritonitis (65). Lastly, CAPD peritonitis has a more benign prognosis than surgical peritonitis.

During peritonitis, the peritoneal clearance of large and small molecules increase while glucose absorption increases. As a result, patients may develop hypophosphatemia, hypokalemia, and hypoproteinemia and also impaired ultrafiltration. Impaired ultrafiltration occurs due to the dissipation of the osmotic gradient in the peritoneal cavity by the absorption of glucose into the bloodstream. Moreover, peritonitis is associated with fibrinogen-rich exudatation and depressed fibrinolytic activity which will result in fibrin clots and further impair drainage (56, 60). Peritonitis can cause significant negative nitrogen balance from a combination of increased protein breakdown and increased protein losses in the PD effluent (64).

Pulmonary complications may occur due to the upward displacement of the diaphragm secondary to bowel distension and by pulmonary edema due to inadequate fluid removal secondary to impaired ultrafiltration.

In certain instances, catheter removal may be required with the temporary discontinuation of CAPD which is replaced with hemodialysis. The catheter is removed in instances of recurrent peritonitis with the same organism suggesting catheter colonization, bacterial or fungal peritonitis not responding to therapy, tunnel infection, intra-abdominal abscess and fecal peritonitis. If the above problems can be overcome, reinsertion of the catheter is possible, otherwise the discontinuation of CAPD becomes permanent.

The progression of peritonitis to a more chronic situation may lead to the formation of a dense layer of fibroconnective tissue on the peritoneal membrane, encapsulating the bowel (i.e. sclerosing peritonitis). Presenting symptoms may include loss of ultrafiltration capacity, recurrent abdominal pain, intermittent vomiting, and eventually, partial or complete small bowel obstruction. This syndrome is a serious complication resulting in a high incidence of fatal outcome. Most deaths result from severe malnutrition, sepsis, and bowel-related surgical complications (82, 144).

As reviewed above CAPD peritonitis is the cause of significant morbidity, of the possible abandonment of CAPD, and in rare instances may result in the demise of the patient.

3. Pathogenesis of CAPD Peritonitis

Despite recent advances, peritonitis remains the leading complication of CAPD. An understanding of the underlying mechanisms of CAPD peritonitis is critical for the prevention of this infectious complication. A large body of

published data supports that numerous contributing factors exist to the development of CAPD peritonitis. For the purpose of exposition in this introductory review, these factors have been grouped under host, technique and bacterial factors.

a) Host Factors

The peritoneum forms the lining boundary of the peritoneal cavity. In a normal individual the peritoneal cavity consists of a space containing 1-20 ml of fluid acting as a lubricant. This fluid has been shown to contain both immunoglobulins of the IgG classes and C3 and C4 components of complement and to opsonize foreign particles (2, 152). The fluid also contains several other constituents of the humoral defense system including lysozyme, fibronectin and basic polypeptides. In patients not undergoing CAPD, the inflammatory response in these patients comprises of an outpouring of polymorphonuclear leukocytes (PMN) into the peritoneal cavity. A delayed response comprises of immunogenic stimulation with increased production of antibodies and phagocytic cells (2, 82, 149). In patients undergoing CAPD this complex system of local host defense may be significantly impaired by several factors, principally the renal failure, some defined aspects of the clinical management of renal failure and the CAPD procedure per se (129, 130).

Renal Failure

Renal failure has long been considered to be a state of immunosuppression responsible for the increased incidence of infections reported in ESRD patients treated with dialysis whether hemodialysis or CAPD. Early investigations in severely uremic patients before the initiation of dialysis have shown the presence of a number of immunological deficits affecting cell-mediated immunity particularly. Several studies have documented the preservation of the mitogenic responses of T-cells in many patients, whereas

responses in mixed lymphocyte cultures and delayed-type hypersensitivity tests were universally depressed (24, 85, 96, 97, 99-101). More recent studies however have not consistently found major immunological deficits in well-dialyzed ESRD patients. It remains difficult to ascribe any given impairment to the renal failure per se because of the many additional factors present in these patients which may also affect immune responses.

Malnutrition may play an important part in the immunosuppression observed in renal failure (1, 12, 41, 140). Quantitative assessments of nutritional status have consistently shown dialysis patients to be malnourished. This state of malnutrition may be clinically overt but most often is subclinical and goes unrecognized. Malnutrition may be a consequence of multiple factors including disturbances in protein and energy metabolism and poor food intake because of anorexia, nausea and vomiting caused by uremic toxicity. With maintenance dialysis therapy, some but not all of these factors are corrected.

The disease which had originally affected the kidney causing the renal failure may also impair immune responses. For instance, approximately a third of CAPD patients are diabetics. Diabetes mellitus is the prime example of a systemic illness with a major impact on the immune system in addition to the expected abnormalities in glucose metabolism (80). Hyperglycemia is known to cause decreased phagocytosis, decreased diapedesis and a decrease in intracellular killing by PMNs. Furthermore, metabolic pathways are altered in diabetic PMNs, which may cause an impaired ability to effectively clear infectious organisms. Lastly, because of the fundamental microvascular disease present in diabetic patients, there may not be an optimal delivery of the host defense cells to the site of bacterial contamination.

Clinical Management

Several aspects of the current clinical management of ESRD patients may also have an adverse effect on immune functions. These patients undergo frequent surgical procedures principally to secure dialysis access and receive frequent blood transfusions because of their severe anemic state. Furthermore, they are often prescribed medication such as steroids and anti-inflammatory agents. All of the above measures are well known to affect immune responses (15, 46, 124, 145, 156).

CAPD Procedure

The procedure of CAPD in itself may pose by itself an additional threat to host defenses. The peritoneal defense system depends largely on two basic mechanisms. Bacterial elimination via the intraperitoneal circulation which carries the bacteria toward the stomata on the surface of the diaphragm where they are transported to the lymphatics and ultimately into the blood stream (78). The in situ elimination of bacteria occurs by the resident phagocytic cells which are mainly mononuclear prior to the incidence of peritonitis which then change to a predominantly polymorphonuclear cell population (131).

First and foremost, the PD solutions represent a constant formidable challenge to the function of the local host defense system of the peritoneal cavity (2). The majority of currently available PD solutions are hyperosmolar, using dextrose as the osmotic agent, and have a low pH, with lactate as buffer. The characteristics of hyperosmolarity and acidity of PD solutions, either alone and in combination, have been shown to impair immune functions in many studies of cell survival and function (67, 68). Of note, these studies have utilized peripheral blood cells harvested from normal individuals and CAPD patients as well as peritoneal cells from stable uninfected CAPD patients. No comparable

studies exist to date utilizing peritoneal cells from normal subjects and infected CAPD patients.

Phagocytic and bactericidal activity are inhibited by hyperosmolar dextrose-based PD solutions. There has been much controversy on the exact nature of the component(s) of PD solutions responsible for the depression of phagocytic and bactericidal activity. DeFijter and colleagues have demonstrated that peritoneal macrophages have decreased phagocytic responses when exposed to 4.25% dextrose solution as opposed to 1.25% (35). However Liberek and colleagues have recently shown that inhibition of phagocytosis is caused by the hyperosmolarity rather than the effect of the increased load in dextrose (87, 88).

The pH and lactate of PD solutions significantly suppress many functions of peripheral and peritoneal leukocytes which include depressed phagocytic and bactericidal activity and leukotriene synthesis (2, 13). Low pH in the presence of lactate buffer was also shown to inhibit production of IL-6 and TNF ∂ by peripheral and peritoneal mononuclear cells (17).

Apart from the direct toxicity of PD solutions on local host defenses, other factors of the PD exchange procedure have a potential impact on the immunological integrity of the peritoneal cavity. The large intraperitoneal fluid volume in CAPD (2 liters) markedly increases the volume/surface area ratio causing further reduction in bacterial elimination by disrupting the normal intraperitoneal fluid circulation (152). The drainage of the peritoneal fluid at regular intervals decrease the efficiency of bacterial elimination by depleting the peritoneal cavity of its immune reactants, both cellular and humoral in nature.

Lastly, several factors directly pertaining to CAPD can aggravate the established nutritional deficits of ESRD patients with potential serious consequences on host defenses leading to increased susceptibility to infection

(1, 12, 140). Metabolic and nutritional problems are accentuated in CAPD patients because of the large loss into the dialysate effluent of proteins, amino acids, vitamins and other essential small solutes (81). These losses are markedly increased during peritonitis episodes (4 to 10 fold). Moreover, peritonitis is associated with absorption of large quantities of dextrose from the dialysate which may lead to hyperglyceridemia, hyperinsulinemia, carbohydrate intolerance and obesity in some patients. Furthermore, the continuous presence of PD fluid in the peritoneal cavity leads to the distension of the abdominal cavity resulting in a decrease of appetite.

b) Technique Factors

The presence of the permanent indwelling PD catheter as a foreign body, the impact of PD solutions on local host defenses and the potential contamination through the delivery system constitute the main aspects of the CAPD procedure recognized as predisposing factors to CAPD peritonitis.

Peritoneal Catheter

For the purpose of CAPD, the required constant access to the peritoneal cavity is provided by the indwelling peritoneal catheter. In bridging the peritoneal cavity to the exterior, the peritoneal catheter necessarily breaks the skin surface which is one of the main barriers of the body against the invasion by infectious agents.

Following implantation, the catheter is subjected to mechanical stresses related to actual and potential catheter movement making it quite difficult for wound healing. If the catheter is inserted with little damage to the surrounding skin, the healing process can occur more effectively creating an airtight seal. Often there is disruption of the seal due to the mechanical stresses resulting in the tearing of the seal, giving access to bacterial penetration and thus leading to opportunistic infections.

Moncrief and colleagues have demonstrated that prolonged subcutaneous implantation of the external segment of the catheter before exteriorization significantly reduces the incidence of peritonitis in CAPD patients (98). This could be ascribed to the fact that the technique allows for wound healing and tissue ingrowth into the cuffs of the catheter promoting the airtight seal.

Persistent presence of infections as a complication of surgical procedures such as the implantation of the permanent indwelling catheter in CAPD patients has been well documented (124). Host-opportunist interaction may occur to affect events at the site of surgery. The absence of local blood supply significantly influences the risk of infection by preventing delivery of the components of the host defense system and similarly the delivery of systematically administered antibiotics. Nonviable tissue surrounding the site of the catheter implantation also significantly increases bacterial virulence. Necrotic tissue provides an anaerobic environment that favours the facultative pathogen, but impairs the phagocytic cell that requires an oxygen-rich environment for proper function. Additionally, the decomposition of dead tissue may serve as substrate for bacterial growth.

Other roles of the catheter as a promoter of infection include an immunosuppressive effect exerted by the presence of a foreign body and it may also provide as the template for bacterial colonization (20, 21, 33, 37, 38, 63).

Several experimental models have been used to assess the role of host factors in foreign body infection. In a seminal study, polymethylmethacrylate tissue cages were implanted subcutaneously in guinea pigs in order to study the effect of the foreign body on phagocytosis by PMNs (163). The overall phagocytic-bactericidal activity of PMNs drawn from the sterile tissue cage fluid was deficient, thus explaining the uncontrollable multiplication of any bacteria

introduced into the experimental model. In a further study, it was shown that PMNs derived from the tissue cage fluid were unable to kill catalase-positive organisms such as S. aureus, but they still possessed some bactericidal activity against catalase-negative bacteria. This finding would suggest that their oxygen-dependent killing mechanisms were defective (i.e. superoxide production was markedly reduced). This set of elegantly detailed observations formed the basis of the hypothesis of impaired phagocytes ("frustrated/exhausted") along non-phagocytosable surfaces (83).

Peritoneal Dialysis Solutions

The non-physiological characteristics of peritoneal dialysis solution has led to the investigation of further modifications in the formulation of PD solutions while still maintaining their capacity for adequate ultrafiltration but attempting to reduce their detrimental actions on peritoneal phagocytic cells and mesothelial cells (13, 40, 152). The osmotic agent, electrolyte and pH have all been manipulated in order to form a more physiological PD solution.

Dextrose is the most commonly used osmotic agent in PD solutions. However, it may no longer be the ideal osmotic agent of choice. This is due to the fact that it is rapidly absorbed across the peritoneum which results in a shorter duration time in ultrafiltration. Moreover, it has adverse metabolic consequences such as hyperglycemia, hyperinsulinemia, hyperlipidemia. It is also damaging to peritoneal mesothelial cells in vitro, particularly inhibiting the cellular proliferation which could interfere with the normal healing process of remesothelization of the peritoneal membrane following an episode of peritonitis (88).

Various osmotic agents have also been assessed for their biocompatibility and maintenance of adequate ultrafiltration.

Low molecular weight osmotic agents (glycerol, sorbitol, amino acids etc...) have been used to overcome some of the metabolic problems of dextrose. When low molecular weight agents are added to PD solutions in the same concentrations as the standard dextrose PD solution, these lower molecular weight osmotic agents have higher osmolarity and produce higher initial transcapillary ultrafiltration rates. However, the peritoneal diffusive permeability of an osmotic agent is also determined by its molecular weight. Low molecular weight osmotic agents are more rapidly absorbed from the dialysate and the transperitoneal osmolar gradient is dissipated more rapidly (91).

Higher molecular weight osmotic agents have been used for both the improvement of the ultrafiltration profile and the minimization of metabolic consequences due to the fact that these agents are not readily absorbed (91). However, higher concentrations are necessary to produce a comparable osmolar gradient which may result in high solution viscosity and reduced dialysate inflow and outflow.

Amino acids were first added to PD solutions in 1968 (55). It has been shown that a 1.1% amino acid solution does not inhibit neutrophil oxidation metabolism. This finding has been attributed to the elevated pH of 6.2 of this formulation rather than to the presence of amino acids. This observation suggests that amino acid-based PD solutions or any solution formulated at a more physiological pH will allow for the host defense system to operate more effectively. Amino acids have also been shown to decrease the rate of proliferation of mesothelial cells in vitro (71).

Glycerol-based PD solutions inhibit the proliferation of mesothelial cells to a lesser extent than dextrose-based PD solutions, thus permitting the remesotheliazation of the peritoneal membrane following an incidence of peritonitis. However, limited studies have demonstrated a greater antagonistic

effect on phagocytic activity in glycerol-based PD solutions when compared to dextrose-based PD solutions (68).

Jorres and colleagues have observed improved cytokine and leukotriene production by stimulated mononuclear cells and PMNs, respectively, in the presence of a glucose polymer-based PD solution as opposed to the standard glucose-based PD solution (76). However, leukocytes and mesothelial cells viability and function were similar in both solutions.

In the past, aluminium gels were the standard oral phosphate binders for dialysis patients with universal hyperphosphatemia. Since these patients are deficient in vitamin D resulting in hypocalcemia, high calcium concentrations were included in the formulation of PD solutions in an attempt to raise serum calcium levels to normal. Aluminum gels have now been recognized as detrimental to health (aluminum intoxication), and have been replaced by calcium-containing phosphate binders. Hypercalcemia is a major side effect of these oral calcium preparations. There are now PD solutions available with low calcium concentrations in order to decrease or reverse the positive peritoneal calcium flux, thereby reducing the incidence of hypercalcemia.

The standard-dextrose based PD solution contains lactate as its buffer which creates an acidic pH causing it to be quite harmful to peritoneal host defense mechanisms as well as peritoneal mesothelial cells in vitro (70). A bicarbonate-buffered solution would be ideal but its manufacturing has created many problems since calcium and magnesium carbonate would precipitate during the sterilization process. Moreover, with the more physiological pH attained, by the introduction of bicarbonate to the PD solutions, the heat of sterilization caused caramelization of dextrose.

A method using a two-chambered bag has been introduced to overcome these problems. The two chambers allow for the separation of the bicarbonate

from the dextrose-based PD solution until the moment of the instillation of the solution into the peritoneal cavity. Short-term studies on the effect of this PD solution have been completed with encouraging results (72).

Until more evidence is forthcoming that any single parameter or combination of parameters of the local host defense system (phagocytes, mesothelial cells, and other immune reactants) is of greater importance to the patient outcome than any other, it will remain difficult to select the appropriate PD solution for therapeutic use.

Delivery system

Over the past several years CAPD has gained increasing popularity due to a dramatic reduction in the incidence of peritonitis (Figure 3). This reduced rate of peritonitis has been ascribed to improvements in sterile technique at the time of exchange and connector technology. The conversion from bottled to bagged dialysate, incorporation of antiseptics such as proviodine into connector devices, oral and intraperitoneal administration of antibiotics, ultraviolet irradiation of the spike-bag junction, and particularly the introduction of the Y-set connectors have led to a remarkable reduction in peritonitis rates (42, 93).

The Y-set contains two limbs with one end attached to a drainage bag and the other end connected to a fresh dialysis bag. The remaining end is attached to the peritoneal catheter extension. In the beginning of an exchange and after the connection of a new bag, a small volume of the fresh dialysis solution is used to flush the system by draining into the drainage bag to wash out any bacteria that accidentally may have been introduced into the system during the connection, thus maintaining the sterility of the procedure.

In spite of experiences gained by nursing personnel of CAPD training units and sustained effort to teach strict aseptic procedures to the patients,

peritonitis still persists in the best centers at a rate around one episode per 12-18 patient months.

c) Bacterial factors

Etiology

CAPD peritonitis is caused by a wide variety of bacterial pathogens, predominantly skin-borne organisms. The clinical manifestations of peritonitis vary depending on the nature of the causative pathogen so that the clinical picture can serve as a clue to the nature of the offending organism. As outlined in Table 1, a typical CAPD peritonitis episode is mild in nature, usually not requiring hospitalization, responding promptly to therapy. Occasionally a more severe presentation occurs with systemic toxic manifestations and marked local changes. Refractoriness to treatment with the development of local complications may be seen in this situation. Lastly CAPD peritonitis has a high incidence of relapse. It is the demonstrated resistance to therapy and recurrence of CAPD peritonitis which renders the management of this condition so challenging.

The exposure of the medical device to the skin flora which takes place when the device is implanted, left in an open transcutaneous wound or manipulated in situ, may explain the predominantly gram positive, coagulase-negative staphylococci (C-NS) infections of medical implants. A small number (4-8%) of peritonitis episodes are caused by fungi, most of them belonging to the Candida species. The C-NS originating from the skin reach the peritoneum by transluminal or periluminal routes. S. epidermidis is the leading cause of peritonitis among the C-NS species (48, 90, 110, 155).

Amongst the staphylococci S. aureus causes a more alarming infection. Patients with this infection may develop a hypotensive state possibly leading to shock. The infection does respond to prompt antibiotic treatment but less

effectively than the S. epidermidis infection with a tendency to abscess formation (150).

Streptococci cause a milder form of peritonitis. The organism most frequently causing peritonitis in this species belongs to the alpha-hemolytic group. The route of infection is believed to be by hematogenous spread as well as intraluminal infection from the oral flora. Other streptococcal species, such as enterococci, may cause peritonitis which would indicate transmural penetration in view of their fecal origin.

Diphtheroids and Propionibacteria are among the least common cause of peritonitis representing 1-2% of organisms isolated from peritonitis episodes (119).

Peritonitis caused by gram negative organisms occur mainly by direct fecal contamination with very few episodes caused by organisms originating directly from the skin. The prevalent gram negative organisms causing peritonitis include E. coli and Pseudomonas aeruginosa. Pseudomonas infections are usually more resistant to treatment often causing multiple abscesses (142). Patients with these infections can develop septic shock requiring management of the hemodynamic disturbances as well as prompt removal of the catheter.

Single episodes of peritonitis caused by a broad variety of microorganisms (e.g. Haemophilus, Neisseria) have been reported indicating that most organisms have the potential of causing infection when penetrating the peritoneal cavity of CAPD patients.

Routes of Entry

The most common route of entry into the peritoneal cavity is by lumen of the peritoneal catheter. Intraluminal contamination usually occurs during connection of the peritoneal dialysis bag port to and from the connecting device

used for injecting the dialysate into the peritoneal cavity. Although the patients are well trained in the procedure, accidental contamination of the dialysate tubing is still possible due to human error. Contamination of the lumen of the connecting device can result from direct contact of the fingers with the connector. Airborne contamination by infected skin scales has also been suggested as a way of bacterial entry in the lumen of the connecting device (34). Many skin scales contain viable bacteria and are attracted to plastic surfaces by electrostatic charges (69). Finally, the penetration of microorganisms into the lumen of the dialysate delivery system can be due to defects and small cracks in plastic connectors, malfunctioning clamps or to faulty PD fluid bags.

An important route of infection of the peritoneum from the skin surface is from the exit site through the subcutaneous catheter tract (periluminal route). This is due to the fact that the catheter never forms a complete seal with the skin, permitting bacteria to establish themselves in the exit site or the tunnel. The catheter has cuffs to increase the stability of its contact to the skin which reduces bacterial penetration.

Less frequent routes of entry are transmural, hematogenous and (in women) ascent through the genital tract. The finding of multiple bacterial species is indicative of bowel wall penetration secondary to a lesion of the intestine.

Transmural contamination results usually from an associated pathology involving the bowel or other organs. Diverticulosis of the colon increases the risk that a patient treated with CAPD will develop peritonitis (161). Many acute viral diseases have also been shown to cause peritoneal infections during CAPD, as have diverticulitis of the colon, appendicitis, perforation of the bowel and acute ischemic colitis (144, 158, 161).

Presence of a Foreign Body

Many surface modifications occur to medical devices in vivo. Foreign bodies are exposed to body fluids and inflammatory cells which lead to the adsorption of host proteins onto the surface of the device. This new coated surface can be used by the bacteria as specific receptors for microbial attachment (21). The presence of host proteins on the medical device can explain the reason for the same susceptibility to infection by the same microorganisms on a variety of different medical devices.

An important role has been ascribed to fibronectin in the bacterial attachment to medical devices. Studies have shown that the ability of bacteria to adhere is greater for implanted foreign material than for non-implanted material demonstrated by the increased attachment of S. aureus to implanted foreign bodies via a direct attachment to surface-adsorbed fibronectin (21).

The deposition of fibronectin onto the catheter is believed to occur by the following mechanisms: 1) Fibroblasts may contribute by their own protein synthesis machinery to the deposition of fibronectin on the extracellular matrix coating the artificial material. 2) Fibrin clots formed on the surface of blood exposed biomaterials contribute to the deposition of fibronectin which is known to be covalently linked to fibrin.

4. Staphylococcus epidermidis

Members of the genus Staphylococcus are considered to be non-motile, catalase-positive organisms that characteristically divide in more than one plane to form clusters of cocci (53, 57). In clinical microbiology laboratories, staphylococci are differentiated primarily by their capacity to produce the enzyme coagulase. Coagulase-positive staphylococci comprise a fairly uniform

species whereas C-NS have been subdivided into 11 species. The relatedness of all staphylococci at the genus level has been confirmed by their similar DNA content (i.e. cytosine and guanine); their divergence into separate species has been ascertained by examinations of specific DNA sequence homology such that less than 5% DNA sequence homology has been designated as a separate species (57).

C-NS are resident, indigenous to the mammalian host and are natural inhabitants of human skin. S. epidermidis is the most prevalent and persistent species on human skin and mucous membranes, comprising from 65-90% of all staphylococci recovered.

Epidemiologic investigations of C-NS have been difficult due to the absence of reliable markers with which to accurately identify the isolates. Antibiotic susceptibility determination, phage typing and biotyping all lack sensitivity and specificity (110).

Recently, staphylococcus organisms have been recognized as an important cause of hospital-acquired infection (8, 154). Infections caused by these organisms involve foreign bodies and are increasing as the number of catheters and artificial devices inserted through the skin become more numerous. These infections are characterized by their indolence but may require the removal of the catheter or the device (25, 51). Resistance of the infections to antibiotic therapy and host defenses has prompted more interest in the characterization of C-NS.

S. epidermidis was not usually considered to be an important pathogen in immunosuppressed patients until reports from two large cancer centers identified it as the most common cause of bacteremia among patients receiving immunosuppressive therapy in their hospitals (155, 159). Winston and colleagues demonstrated that central intravenous catheters were the source for

S. epidermidis bacteremia in their patients (159). Most of these patients were markedly neutropenic. Subsequent studies have reconfirmed indwelling catheters as the source of bacteremia in immunosuppressed patients. Gram positive bacteria, particularly S. epidermidis, accounts for 50-80% of the organisms causing catheter-related bacteremia (89, 121, 139).

5. Clinical Management of CAPD Peritonitis

a) General Measures

At presentation, the clinical diagnosis of peritonitis is based on the presence of two of the following three factors in any combination: 1) Cloudy dialysis effluent containing greater than 100 cells/μl consisting predominantly of PMNs, 2) abdominal pain or tenderness, and 3) identification of microorganisms either on gram stain or culture directly from spent PD fluid (42, 116, 138).

Once the diagnosis of peritonitis is suspected, several steps of management are taken prior to the final assessment of gram stain and/or culture and antibiotic sensitivity.

The initial step in the early treatment of peritonitis consists in a series of rapid PD exchanges allowing for the removal of mediators of inflammation from the peritoneal cavity, thus leading to the relief of abdominal pain. The subsequent step in treatment is the instillation in the peritoneal cavity of fresh dialysis solution admixed with the appropriate antibiotics to cover a broad range of pathogens possibly involved in causing the peritonitis. Heparin is usually administered to reduce the formation of fibrin clots and prevent subsequent adhesion formation (47). This regime is followed by other exchanges with the same above mentioned additives at reduced concentrations until the results of

the gram stain and/or culture are available. Appropriate changes are then made if necessary according to the nature of the causative pathogen.

b) Antibiotic Treatment

The intraperitoneal (i.p.) route is often the preferred route for the administration of antibiotics. The i.p. route allows the antibiotics to gain direct access to the area of infection at proper bactericidal concentrations; they are readily reabsorbed from the peritoneal cavity and can achieve adequate serum levels, and finally, they are easily administered by the patients. The procedure of antibiotic administration usually includes the instillation of a fresh PD solution containing a loading dose of antibiotics to obtain good serum levels, whereas subsequent exchanges contain a lower maintenance dose to prevent serum antibiotic accumulation and potential toxicity.

The antibiotics selected for initial treatment should be effective against the most frequent organisms observed in peritonitis while providing coverage for rare organisms. First choice of antibiotics should cover most gram positive organisms as well as gram negative organisms. Most centers use a cephalosporin (i.e. commonly cefazolin) which has a good coverage for S. epidermidis and S. aureus while also covering a certain number of gram negative organisms. The addition of an aminoglycoside will cover more threatening enteric organisms (i.e. large spectrum of gram negative organisms). Cefazolin is replaced by vancomycin in situations where the patient is hypersensitive to cephalosporins or in instances of contamination by methicillin-resistant staphylococci. Once the organism has been identified and an antibiotic sensitivity is available, adjustments are made in the choice of antibiotics.

The effective length of treatment is approximately 2-3 weeks. If no clinical improvement and/or decrease in cell count is evident after 4-5 days,

repeat cultures are necessary and a change in the choice of antibiotics is considered.

Table 2 reviews the current recommendations for antibiotic therapy of peritonitis when S. epidermidis is the documented or possible causative pathogen. The Table emphasizes the extensive clinical use of vancomycin, aminoglycosides and rifampin.

There are many side effects to the use of antibiotics. Eosinophilia in the peritoneal fluid may be observed and skin rashes may occur. Aminoglycosides are known to have nephrotoxic and ototoxic effects as well as vestibular toxicity. Rifampin occasionally results in the elevation of liver enzymes or gastro-intestinal intolerance, necessitating the discontinuation of the drug. Because of these possible side effects, strict monitoring of available antibiotic levels is necessary.

6. Recent Insights into the Pathogenesis of Infections Associated with Medical Implants: Bacterial Biofilms

General Considerations

CAPD peritonitis is a prime example of an infection associated with medical implants. Table 3 presents the characteristic clinical features of this type of infections. These infections are predominantly due to S. epidermidis, a skin commensal of limited pathogenicity in the absence of implanted material, and are characterized by an indolent course (evidence of resistance to host defenses) and poor responses to antibiotic therapy.

The resistance of implant-associated infection to antibiotic therapy has been ascribed to the properties of the bacteria, usually S. epidermidis, to adhere readily to artificial surfaces and produce a protective coating of a

polysaccharide matrix or slime to form a bacterial biofilm exerting a selective or absolute impermeability to harmful agents of the environment such as antibiotics (27, 32, 86, 125, 126, 153). Slime producing strains of bacteria may also cause persistent and recurrent infection associated with medical implants by impeding the penetration of phagocytic cells and humoral immune factors (29).

In the early 1960s staphylococcal taxonomist Baird-Parker Jones noted the production of slime by C-NS (75). In 1972 Bayston and Penny reported the presence of slime material on the surface of ventricular shunts removed from children with cerebrospinal fluid shunt infections (9). This first observation suggested that slime material may be important in the pathogenesis of infections associated with medical implants.

Christensen and colleagues were the first to report the high incidence of intravascular catheter-associated sepsis due to C-NS (23). These researchers were interested in slime production as a possible mode of attachment of staphylococci to solid surfaces. In a series of studies comparing the virulence of a slime producing strain with the virulence of a daughter strain that did not produce slime, they established that slime was a virulence factor (22). The virulence of the non-slime producing strain was reduced by one third when compared with the parent slime producing strain. Further studies demonstrated that slime production was associated with in vivo and in vitro resistance to antimicrobial agents (36, 129, 143, 162).

Microbial cells attach firmly to almost any surface submersed in an aquatic environment. These immobilized cells can grow, reproduce and many produce extracellular polymers which frequently extend from the cell forming a matrix which provides structure to the assembly termed a biofilm. This extracellular polymer appears as a highly hydrated capsule almost exclusively

made of polysaccharides. Biofilms are not restricted to a certain environment, they are associated with pipes, dental plaque, cholera and other intestinal diseases and many more.

The majority of biofilm matrices are exopolysaccharides (EPS). Based on their chemical composition, antigenic specificity, and mode of biosynthesis, the EPS are divided into specific and non-specific polysaccharides (114). Specific polysaccharides are specific to individual bacterial strains. Common saccharides such as glucose, galactose, mannose, N-acetyl glucosamine, glucuronic acid and galacturonic acids are typical constituents of specific polysaccharides. The non-specific polysaccharides are found in a variety of bacterial strains and are structurally different, for instance they may contain only one monomer.

The exact chemical structure of the EPS of C-NS is still unknown; however, there is some evidence that the EPS is rather a complex glycoconjugate and not a carbohydrate polymer. Traces of glucose, galactose, mannose, glucosamines and glucuronic acid have been identified in the EPS of C-NS (114).

Colonization Process

The process of bacterial colonization with slime production begins with the exposure of the implanted device to bacteria and the inevitable attachment through non specific binding of the bacterium to the implant. The precise number of bacteria attaching to the surface varies depending upon the concentration of bacteria surrounding the implant and the specific physiochemical properties of the bacteria and the surface (21, 28, 112, 113, 146).

Local conditions can facilitate bacterial adhesion to the device. All foreign bodies exhibit microscopic surface irregularities which create unique

microscopic sites which allow or even promote bacterial attachment. These sites include microscopic structural defects in the surface, local variations in the physiochemical characteristics of the surface and the deposition of host protein receptors on the surface (21).

Slime production promotes the accumulation of the bacteria on the medical device by providing for the integrity and positional stability of the microcolony. Therefore, the ability of the biofilm to be produced can permit the bacteria to convert the inhospitable surfaces of all medical devices into a receptive surface for bacterial colonization (9, 30, 61, 74).

Effects on Host Defenses

Implant-associated infections with S. epidermidis as the main pathogen are frequently indolent in course, suggesting a reduced mediated inflammatory response to the organism. Several in vitro investigations have demonstrated that slime matrix interferes with many immune functions. It is noteworthy that these observations were made using various preparations of EPS extracted from bacterial cultures, with the possible contamination with bacterial components and products as well as elements of the growth culture medium.

Johnson and colleagues have investigated the effect of the glycocalyx matrix on PMN chemotaxis and phagocytosis (111). They have demonstrated that the preincubation of PMNs with the glycocalyx matrix produced by S. epidermidis inhibited the chemotactic responses of the cells to an added known chemoattractant, zymosan-activated serum. The results demonstrated that the glycocalyx matrix interacted with PMNs to alter their function without affecting their viability. The phagocytic activity of PMNs was also assessed in these studies examining the adherence and growth of S. epidermidis on plastic plates containing proper nutrients for the production of slime. The results

demonstrated a marked reduction in the uptake of the bacteria by PMNs compared to the control bacteria with minimal slime production (111).

Gray and colleagues have demonstrated that the proliferative response of circulating lymphocytes by polyclonal T cell stimulators was inhibited by the presence of slime (59). The mechanism of action can be in part explained by a gradual lytic action of peripheral blood mononuclear cells.

Moreover, the glycocalyx matrix also inhibits the cytotoxic activity of the natural killer (NK) cells and it also alters the surface antigens of lymphocytes (59, 111). The surface modifications of the lymphocytes will inhibit the functional ability of T helper cells which alters the formation and release of lymphokines necessary to stimulate the proliferation of other lymphocytes.

In summary, the effect of the components of the glycocalyx matrix on host defenses may in part be responsible for the ability of this avirulent microorganism, S. epidermidis, to become pathogenic and persist and thrive in implant-associated infections.

Resistance to Antibiotics

The hydrated exopolysaccharide matrix that encloses the bacterial cells within an adherent biofilm constitutes a polymeric and predominantly anionic material that allows the influx of nutrients to the bacterial cells but severely limits penetration of biocides, antibodies and antibiotics (27, 62).

Costerton noticed that the antibiotic susceptibility of the planktonic bacterial cells liberated from the protective microcolonies in the biofilm is similar to that of the unprotected bacterial cells in in vitro cultures, whereas the bacterial cells within the biofilm were demonstrated to be more resistant to many conventional antibiotics (26).

Peters and colleagues performed MIC determinations for three different Staphylococcus strains against several antibiotics using the Mueller-Hinton

(MH) broth as the standard laboratory milieu and pmS 110-broth which promotes slime production (114). Using an inoculum of 10^7 cfu, a dramatic change in the antibiotic susceptibility was observed, from being susceptible in MH-broth towards being completely resistant in pmS 110-broth. The MIC values in pmS 110-broth were determined to be more than 15 times higher than those in MH-broth, thus attributing the increased resistance to the presence of the slime.

Bacterial Biofilms on CAPD Catheters

Bacterial biofilm colonization on the permanent indwelling catheters is very common in patients undergoing CAPD treatment. Dasgupta and Costerton have demonstrated extensive bacterial biofilm developing over time on both the extraluminal and intraluminal surfaces of the peritoneal catheters (33). Surprisingly they found similar biofilm formation on the catheters from all CAPD patients whether they had peritonitis or not. In some of the patients free of peritonitis, bacterial biofilms were observed on catheters left unused in situ for 3 months. These findings suggest that bacterial biofilm colonization on the catheters of CAPD patients is inevitable over time. It is suggested that failure of host defense mechanisms, or the dialysis procedure itself may lead to the dissemination of bacteria from the biofilm present on the catheter surface and result in the development of peritonitis.

Current Methods of Morphological Study of Bacterial Biofilms

The application of light microscopy, electron microscopy, image analysis and computer-enhanced microscopy to studies on bacterial attachment and biofilms has provided information on morphology and bacterial cell density of biofilms, adherence properties and responses to environmental stress.

Many investigators have demonstrated the colonization of foreign bodies by bacterial biofilms using coupled transmission and scanning electron

microscopy (TEM/SEM) (94, 95, 113). These techniques have provided a wealth of information on the morphology of bacterial biofilms in nature and disease. However, biofilm preparation for TEM/SEM induces considerable morphological changes (dehydration, embedding and disruption), leading to estimated shrinkages of 50% during fixation (160). Furthermore, TEM/SEM techniques are laborious and can produce artifacts resulting from the processing. These techniques also are limited in the ability of 3D construction of biofilms.

There continues to be a need for non-destructive procedure for studying the development and structure of undisturbed microcolonies and biofilms on foreign bodies. Epifluorescence techniques have been widely used; however the major disadvantage to these techniques (and other light microscopy methods) is the degradation of the image by out-of-focus information originating from focal planes above or below the specimen (157). The development of scanning confocal laser microscopy (SCLM) offers detailed visualization of thick microbiological samples without the dehydration process observed in TEM/SEM. SCLM allows optical and sectioning (0.2 μm intervals) in both the horizontal and vertical planes and the elimination of out-of-focus haze. Lastly SCLM enables the determination of 3D relationships of cells and 3D computer reconstruction from optical thin sections.

Lawrence and colleagues have produced the first images of intact, fully hydrated biofilms of P. aeruginosa, P. fluorescens, and Vibrio parahaemolyticus using SCLM (86). Horizontal and sagittal sections of intact biofilms were analyzed by image-processing techniques to assess the distribution of cellular and noncellular areas within the biofilm matrices. Between the two bacterial species examined, Pseudomonas biofilms were most cell dense at their attachment surfaces and became more diffuse near their outer regions,

whereas Vibrio biofilms demonstrated the opposite trend. Biofilms of different species demonstrated distinctive arrangements of the major biofilm structural components (cellular and extracellular materials and space). In general biofilms were found to have a high content of water, open structures composed of 73-98% extracellular materials and space.

7. An Assay of Antimicrobial Activity Against Experimental Bacterial Biofilms

Implant-associated infections have demonstrated resistance to host defenses and antibiotic therapy. It has been shown in vitro that this resistance is due to the production of a shielding matrix of polymerized carbohydrates, when the bacteria adheres to the implant, enclosing the bacteria resulting in the impedance of the penetration of therapeutic agents and host defenses. Quantitative experiments on the antimicrobial activity of therapeutic agents have been assessed by many techniques including the measurement of bacterial cell count by light microscopy, chemical measurement of specific constituents of the bacteria, changes in the environment, etc...(18). These techniques are quite complex and time consuming. A recently introduced assay has been used throughout this thesis which allows for more simple and rapid assessments in determining efficient therapeutic regimes against implant-associated infections (128, 135). The assay has enabled the previous demonstration of the superior although incomplete effect of rifampin against S. epidermidis biofilms and the synergistic and antagonistic interactions of rifampin with several antibiotics (48-51, 107, 108).

Table 1. Distinguishing clinical features between surgical and CAPD peritonitis

	Surgical peritonitis	CAPD peritonitis
Frequency	Rare	Frequent
Cause	Bowel rupture	Touch contamination of catheter during dialysis exchange
Route of entry	Gross contamination with intestinal content	Intraluminal/periluminal
Pathogen	Enteric organisms	Indigenous skin organisms
Clinical manifestations		
• Systemic	Highly toxic Fever	Uncommon Usually afebrile
• Local	Universally severe	Limited
Blood Culture	Positive	Negative
Prognosis	Guarded	Relatively good

Table 2. Current recommendations for the treatment of CAPD peritonitis[a].

On clinical presentation with peritonitis	
Gram stain and culture	Treatment
• Gram positive organism	Vancomycin
• Culture negative	Vancomycin and aminoglycoside
After 24-48 hours of treatment	
Gram positive organism on culture	
• Gram positive organisms other than enterococci and S. aureus	Vancomycin (If no improvement add rifampin)
Culture negative	
• If clinical improvement	Vancomycin
• If no clinical improvement	Repeat microbiological assessment
Apparently relapsing peritonitis[b]	
Gram stain and culture	
• Gram positive organism other than enterococci and S. aureus	Vancomycin (If no improvement add rifampin)
• Culture negative	Vancomycin and aminogycoside

[a]Recommendations for the treatment of peritonitis with documented or possible S. epidermidis etiology.
From: The Adhoc Advisory Committee. Peritoneal dialysis-related peritonitis treatment recommendations - 1993 update. Perit Dial Int 13, 14-28, 1993.
In all instances, if no improvement catheter removal may be required.
[b]Defined as another episode of peritonitis caused by the same genus/species that caused the immediately preceding episode and occurs within 4 weeks of completion of the antibiotic course.

Table 3. Features of infections associated with medical implants.

Altered bacterial pathogenicity
• Reduced critical number of bacteria required to initiate infection
• Emergence of avirulent bacteria as pathogenic (S. epidermidis for instance)
Altered host defenses
• Change in course and character of the infection (chronic, indolent)
• Hypothesis of exhausted phagocytes
Altered response to antibiotic therapy (i.e. antibiotic resistance)
• Sequestration of the bacteria by uncontrolled tissue extension
• Sequestration of the bacteria by host proteins on the artificial surfaces

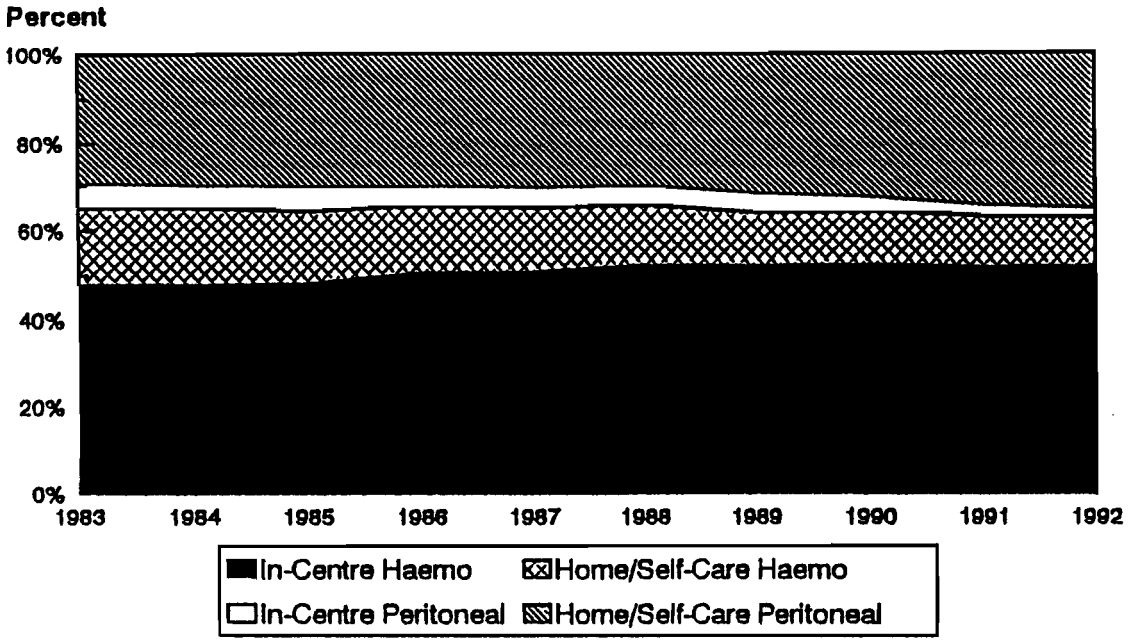


Figure 1. Percentage distribution by type of dialysis, all patients, Canada. Data from the 1992 Annual Report of the Canadian Organ Replacement Register (C.O.R.R), Hospital Medical Reports Institute, Don Mills, Ontario, March 1994, Figure 6, pp 55. Permission for reproduction granted by Dr. Stanley Fenton, Chairman of the Dialysis and Renal Transplant Sub-Committee of the C.O.R.R.

The absolute number of patients on peritoneal dialysis was 1311 in 1983 and 2826 in 1992 (Also from C.O.R.R. 1992, Table 7, pp 54).

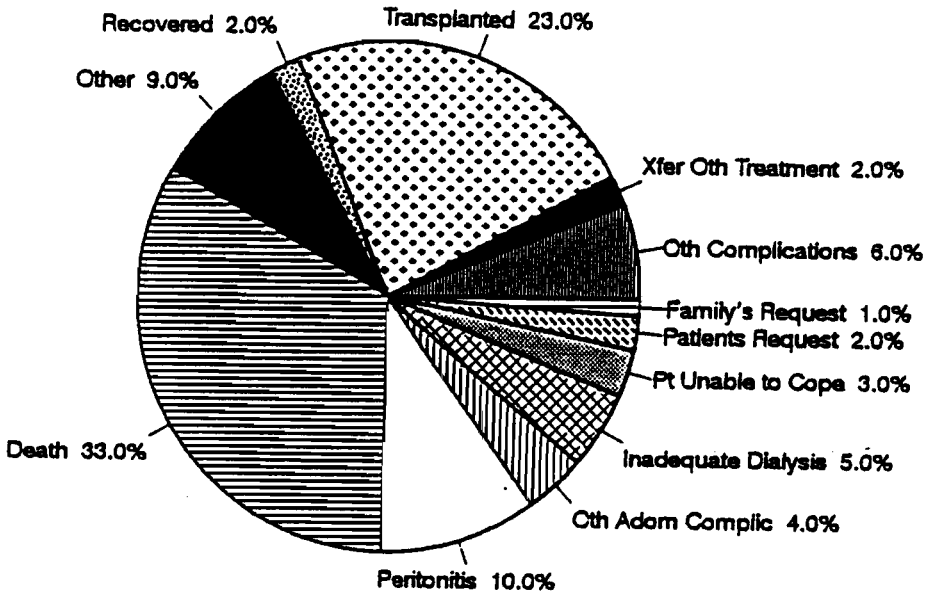
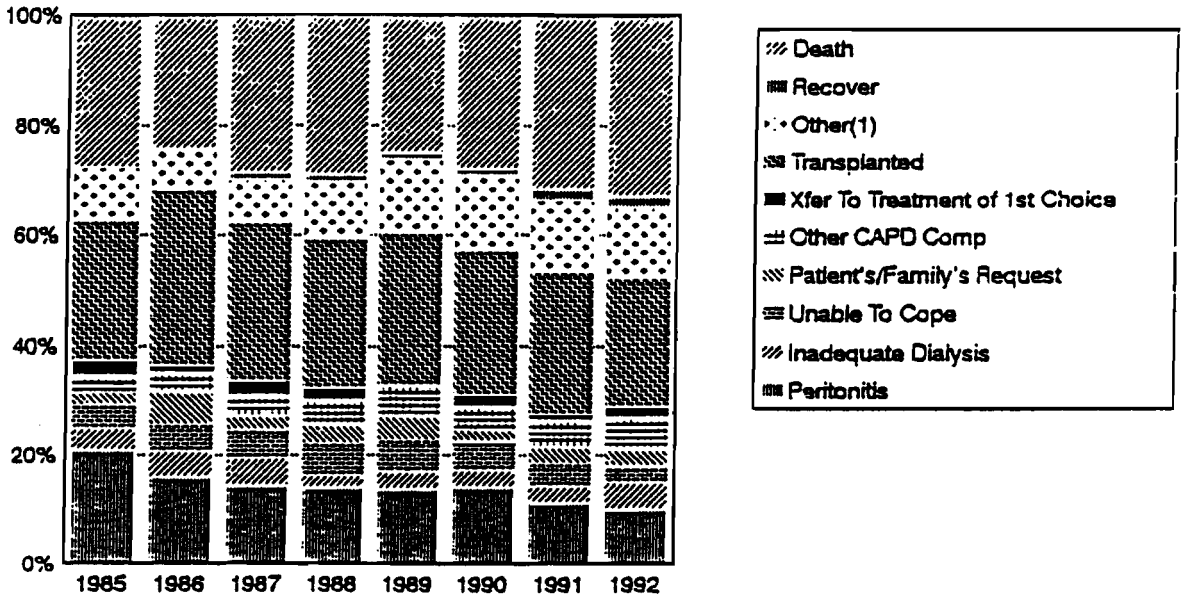


Figure 2. Reasons for discontinuation of CAPD in Registered Patients, Canada, 1992. Data from the 1992 Annual Report of the Canadian Organ Replacement Register, Hospital Medical Reports Institute, Don Mills, Ontario, March 1994, Figure 33, pp 118. Permission for reproduction granted by Dr. Stanley Fenton, Chairman of the Dialysis and Renal Transplant Sub-Committee of the C.O.R.R.



1. Other abdominal complications combined with other reasons.

Figure 3. Reasons for discontinuing CAPD, Canada, 1985-1992. Data from the Annual Report of the Canadian Organ Replacement Register, Hospital Medical Reports Institute, Don Mills, Ontario, March 1994 Figure 34, pp 118. Permission for reproduction granted by Dr. Stanley Fenton, Chairman of the Dialysis and Renal Transplant Sub-Committee of the C.O.R.R.

AIMS OF THESIS

To assess antibiotic activity (concentration/kinetics) against experimental Staphylococcus epidermidis biofilms, utilizing a minaturized assay

1) To determine the relative rates of action of clinically relevant antibiotics.

2) To assess the modulating effect of CAPD variables on antibiotic rates of action (PD solutions and non-antibiotic therapeutic additives to PD solutions).

3) To examine the morphological changes of fully hydrated biofilms following exposure to selected milieus using scanning confocal laser microscopy.

MATERIALS AND METHODS

1. Materials

a) Bacterial Isolates

A clinical isolate of Staphylococcus epidermidis (MGH #2503), obtained from a blood culture of a patient with an implant-associated infection, was noted to be a stable slime-matrix producer. It was characterized in detail and stored in aliquots at -70°C. Aliquots were revived as required, identity and characterization confirmed monthly to exclude strain variation. The isolate was a catalase-positive, coagulase-negative, gram positive coccus growing in clusters. The characteristics of the rifampin-sensitive S. epidermidis by automated Vitek testing are presented in Table 4a. This strain is fully sensitive, by routine test methods in the fluid phase to appropriate antibiotics including rifampin.

This strain demonstrated the abundant production of a glycocalyx matrix (i.e. the biofilm phase) when adherent to surfaces in appropriate culture milieus. The pattern of growth of the S. epidermidis strain in the biofilm phase was examined by gram stain (which demonstrates the bacteria) and toluidine blue staining (which demonstrates the matrix) (106). The adherent bacterial cells on glass and Silastic surfaces were first noted at 3 hours of incubation, followed by the presence of microcolonies after 9 hours of incubation. Extracellular slime production was shown by toluidine blue staining at 14 hours of incubation (106). This slime production was comparable to the matrix formed by two reference strains (RP12 and RP14) kindly provided by Dr. Gordon D. Christensen, Columbia, Missouri, USA, and which are now catalogued in the American Type Culture Collection. After 18 hours incubation, confluent matrix

formation was obtained consistently on glass surfaces under the experimental assay conditions.

A stable rifampin-resistant (mutant) derivative of the S. epidermidis MGH #2503 strain was developed by Dr. Geoffrey K. Richards, Regina, Saskatchewan, Canada, and kindly supplied to this laboratory and for brevity was labelled #2503RR. The derivative strain was identical in all respects to the parent strain except for the gain of lactose fermentation and solid resistance to rifampin. The derivative strain also demonstrated the production of an abundant glycocalyx matrix, although at a lesser rate of production than the parent strain. The characteristics of S. epidermidis #2503RR by automated Vitek testing is presented in Table 4b.

b) Peptone Water

Peptone water (Oxoid Ltd., England) is a standard laboratory medium. It is a peptone-saline digest without fermentable carbohydrates. It is an amphoteric buffer, protective to bacteria, affording a source of energy to maintain bacterial viability but insufficient carbohydrate to permit significant increase of the extracellular polymer substance during the period of tests. It was used as the control medium in all the assays to determine the baseline metabolic activity of the standardized biofilm preparations of S. epidermidis to be compared with the metabolic activity of biofilms exposed to various alternative milieus. Peptone water was additionally used as the standard diluent in the preparation of the required concentrations of the test agents.

c) Growth Media

Standardized reproducible S. epidermidis biofilms were prepared in a nutrient broth which consisted of a mixture of 25% by volume of peptone water and 75% tryptone soya broth (TSB, Oxoid Ltd., England) previously sterilized by autoclaving for 15 min at 121°C. This growth medium contains fermentable

carbohydrates which are required by the bacteria to produce the exopolysaccharide matrix within the initial 18 hours period of incubation at 37°C.

A 100% TSB solution was used as the growth medium for the formation of the exopolysaccharide matrix by the rifampin-resistant S. epidermidis strain (#2503RR) in order to accelerate the production of a glycocalyx matrix to reach a comparable density to that of the rifampin-sensitive S. epidermidis strain (MGH #2503).

d) Metabolic Indicator

Bacterial metabolic activity was assessed by the reduction of 2,3,5-triphenyltetrazolium chloride (TTC, Sigma Chemicals). TTC easily diffuses through the biofilm matrix and bacterial cell wall. At the level of the cell membrane it competes with the electron acceptors in the electron transport chain thereby becoming reduced to an insoluble formazan which is deposited as red granules within the bacterial cell, visible by microscopy (4, 6, 7, 14, 45, 84, 147). The degree of reduction of TTC is directly proportional to the density of the actively respiring bacteria in the culture and is therefore a measure of biofilm viability (135). The development of formazan within a metabolically active biofilm preparation can be detected by eye if the density of viable bacteria is sufficiently high.

A 1% stock solution of TTC in distilled water was sterilized by vacuum filtration and stored at 4°C for later use. Five ml of the stock solution was added to one liter of 2% nutrient agar previously sterilized by autoclaving for 15 min at 121°C and cooled in a 55°C water bath for 30 min. The mixture was mixed and poured in 20 ml volumes into standard sterilized, disposable 100 X 15 mm diameter plastic petri dishes (Fisher Scientific Limited, 8-757-13, Montreal, Canada) and left to cool to room temperature in order for it to harden.

The plates were stored at 4°C in sealed packages for later use within a 2 week period.

e) Glass Slides

Clear soda glass microscope slides (Surgipath 00220, Winnipeg, Canada) were used as the template for S. epidermidis biofilm formation. In previous studies, the adherence and biofilm formation of the strain MGH #2503 to the glass slides was demonstrated to be essentially similar to the indwelling catheters of CAPD patients (106). Therefore, glass slides were utilized throughout.

The slides were individually cut, with a diamond cutter, into glass strips measuring 6 x 25 mm. Surface markings (the letter "F") were etched on one side of the glass strips in order to later identify the side of biofilm growth. For each batch, 25 glass strips were arranged in a glass pyrex petri dish with the surface markings face down the biofilm forming on the upper side and sterilized by autoclaving for 15 min at 121°C.

f) Screw-Capped Vials

Five ml screw-capped vials 15 x 45 mm (cat no. 66011304, VWR Scientific, Canada) sterilized by autoclave for 15 min at 121°C were used to contain the individual bacterial biofilm slides for the period of exposure to the different milieus.

2. Routine Biochemistry

Aliquots of peptone water, fresh PD solutions, individual spent fluids from ten CAPD patients and pooled spent fluid were tested at the Department of Medical Biochemistry of the Montreal General Hospital. A series of biochemical analyses were performed on each sample by autoanalyzer (Model 717,

Boehringer-Manheim Hitachi, Japan). This provided for a range of relevant biochemical tests namely, osmolarity, glucose, sodium, potassium, chloride, bicarbonate, lactate, urea, creatinine, uric acid, total protein, albumin, total calcium, phosphate, magnesium and iron.

The pH of the solutions and biological fluids was determined using a standard digital pH meter (Fisher Accumet, Model #830).

3. Routine Microbiology

The antibiotic susceptibility of both S. epidermidis strains was determined for a panel of antibiotics using standard microbiological procedures. A lawn of S. epidermidis MGH #2503 at the NCCLS standard concentration was prepared on a Mueller Hinton (MH) agar plate by overnight incubation at 37°C with an antibiotic disc at the center of the plate. Confirmation of susceptibility was obtained by a zone of inhibition equivalent or greater than the required NCCLS diameter.

The rifampin resistance of the derivative strain #2503RR was assessed by plating a MH plate with the derivative strain and placing a rifampin disc (5 μg/ml) at the center of the plate followed by overnight incubation at 37°C, confirming solid resistance by bacterial growth to the margin of the disc.

Monthly confirmation of identity (biotype number) and automated susceptibility testing was obtained using Vitek technology.

The antimicrobial activity of the individual spent peritoneal dialysis fluid from ten CAPD patients was assessed by inoculating each spent fluid aliquot onto a MH agar plate plated with S. epidermidis (MGH #2503) then incubated overnight at 37°C. The presence of antibiotic activity was indicated by the inhibition of growth of bacteria by the inoculum of spent fluid.

4. The Bacterial Biofilm Assay

An assay of antibiotic action was developed in this laboratory which assesses the bactericidal action of different milieus against standardized in vitro biofilm preparations of S. epidermidis (MGH #2503). This assay permitted the evaluation of the rate of action and bactericidal potential of individual antibiotics; double and triple antibiotic combinations; the effect of increasing concentration and time of exposure; and the impact of the presence of peritoneal dialysis fluids and interactions with therapeutic additives. As outlined in Figure 4, the bacterial biofilm assay comprised the following three main steps:

- the preparation of the biofilms,
- their exposure to different milieus,
- the subsequent determination of the biofilm residual metabolic activity.

Photographs of selected aspects of the biofilm assay are presented in Figures 5 and 6.

a) Preparation of Standardized S. epidermidis Biofilms on Glass

A loopful of S. epidermidis growth on the blood agar was inoculated into 5 ml of peptone water. The bacterial suspension was incubated at 37°C overnight to achieve a log-phase of growth. Twenty ml of the sterile growth medium (25% peptone water / 75% TSB) was dispensed into petri dishes containing the glass strips (6 x 25 mm). Each dish was seeded by the addition of 0.03 ml (approximately 10^4 cfu) of the overnight culture and incubated for 18 hours at 37°C (Figure 5a). Biofilms formed on the glass strips were regarded satisfactory if achieving an optical density of 3 Mcfarland units and a bacterial density of circa 10^7 cfu/ml.

b) Exposure of the Biofilms to Different Milieus

Following a further 18 hour period of incubation, the glass strips now containing the S. epidermidis biofilms on the upper surface were rinsed three times in sterile distilled water to remove loosely-attached surface free-floating bacteria. The glass strips were then blotted dry on sterile filter paper (Figure 5b).

The S. epidermidis biofilm preparations were individually placed in the screw-capped vials containing 2.5 ml volumes (Figures 5c and 5d). The vials were placed horizontally in racks permitting optimum air/fluid volume ratio to maximize aeration with the biofilm surface of the slides uppermost (Figure 5e). These vials were incubated at 37°C for selected periods of time up to a maximum of 5 days. When longer than 24 hours of incubation was selected, the test medium was changed daily to avoid confounding variations due to exhaustion of nutrients or pH change. Unless otherwise mentioned, the media did not contain any fermentable carbohydrates in order to prevent changes in the biofilm density by further production of the exopolysaccharide matrix.

Each assay contained a positive control of growth, which was the biofilm exposed to peptone water free of therapeutic agents, and a negative control of growth consisting of the biofilm preparation exposed to 4% formaldehyde (Sigma Chemicals) for 4 hours.

c) Assessment of Biofilm Residual Metabolic Activity

After the selected exposure period, the biofilms were removed from the screw-capped vials under sterile conditions and rinsed in sterile distilled water to remove active agents. The biofilm strips were then placed onto the surface of the TTC agar plates with the biofilm side facing down on the gel (Figure 5f). The plates were further incubated at 37°C for 24 hours to permit the full development of the red color of reduced formazan. This color development

indicated the presence of continuing metabolic activity in the biofilm bacteria. The metabolic activity of the exposed biofilms were measured semi-quantitatively by the intensity of the color change of the biofilm using naked eye inspection (Figure 6). The daily grading of the intensity of color ranged from 3+, comparable to the positive control (dark red), to 0 which indicates cessation of metabolic activity. The final results were expressed as time required in days for cessation of the metabolic activity of the bacterial biofilm. Example results are illustrated for 4 commonly used antibiotics in Table 5.

5. Test of Anti-biofilm Activity of Therapeutic Agents

The various therapeutic agents instilled in the peritoneal cavity of CAPD patients were examined (Table 6). They included antibiotics, peritoneal dialysis solutions (both fresh solution and spent fluid) and common non-antibiotic therapeutic additives to PD solutions. The agents were examined singly and in combination. Antibiotics were tested at the standard concentration of 10 μg/ml and, as noted, using a larger range. The non-antibiotic therapeutic additives to PD solutions were tested at the upper range of the recommended concentrations in CAPD patients.

a) Antibiotics

The antibiotics may be divided into 3 groups: 1) Those which primarily result in cell wall defects were termed "cell-wall active". Cloxacillin, cefazolin and vancomycin belong in this group. 2) Those belonging to the aminoglycoside family on the basis of chemical structure were termed the "aminoglycoside group". These include streptomycin, gentamicin, tobramycin, netilmycin, neomycin and amikacin. 3) The remaining antibiotics with various

modes of interference with bacterial metabolism were termed "others". This is represented by ciprofloxacin and rifampin.

Clinically relevant antibiotics were assayed against the standardized S. epidermidis biofilms. Four antibiotics were tested repeatedly in detail throughout this investigation: rifampin, cefazolin, vancomycin and gentamicin. Rifampin has been previously shown to possess a rapid but incomplete effect against S. epidermidis biofilms (108, 133). Cefazolin is known to be an effective anti-staphylococcal agent. Vancomycin is utilized in clinical situations of CAPD peritonitis caused by methicillin-resistant gram positive organisms. Finally, gentamicin is frequently administered to patients with infections associated with medical implants particularly in combination with vancomycin with or without rifampin. These agents were assayed alone, in combination with rifampin and also in the presence of non-antibiotic therapeutic agents (as might occur in clinical practice).

The antibiotic solutions were prepared from commercially available antibiotic susceptibility discs (Oxoid, Unipath Limited) placed in bulk in appropriate volumes of peptone water, 30 min prior to use in order to allow complete elution of the antibiotics from the discs. The effect of varying concentrations of the clinically relevant antibiotics on bacterial biofilms were examined singly and in combination with other antibiotics.

b) Peritoneal Dialysis Solutions

i. Fresh Peritoneal Dialysis Solution

Several fresh PD solutions were examined for their potential modulating effect on the antimicrobial activity of antibiotics against the biofilm preparations. These solutions included formulations which are commercially available worldwide, and others which are undergoing clinical trials or are in limited use in selected dialysis units. The commercially available PD solutions comprised

dextrose-based dialysis solutions with two different calcium concentrations (1.25 and 1.62 mmol/L) and a 1% amino-acid based dialysis solution used especially in pediatric CAPD patients. The PD solutions under clinical trial included a polyglucose-based dialysis solution and a dextrose-based solution containing bicarbonate as buffer. The PD solution under restricted use was glycerol-based. The pharmaceutical source of the solutions is provided in the legend to Table 13.

ii. Spent Peritoneal Dialysis Fluid

The moment PD solution is instilled in the peritoneal cavity it no longer has the same characteristics prior to instillation and is now characterized as spent fluid. Spent fluid was used as an environment to measure the impact on the activity of a number of antibiotics directed against S. epidermidis biofilms.

The spent dialysis fluid consisted of a pool of PD effluents collected after an overnight intraperitoneal dwell with 4.25% dextrose-based PD solution (lactate buffer, Ca^{++} 1.62 mmol/L) in ten stable CAPD patients from the MGH Home Dialysis Program. The patients had been free of peritonitis for the preceding 6 months. At the time of study the patients had not been treated with any antibiotics in the previous 2 weeks nor were drugs added to their fresh PD solution bag. Within one hour of collection, each effluent was centrifuged at 3000 x g for 30 min at room temperature (Beckman, GPR centrifuge) to remove cells and clots and then stored in aliquots at -20°C. All effluents were screened to exclude the presence of antibiotic activity before pooling. Aliquots of the individual and pooled spent fluids were also evaluated by routine biochemistry.

c) Non-antibiotic Therapeutic Additives to Peritoneal Dialysis Solutions

Several non-antibiotic drugs are frequently administered to CAPD patients via the peritoneal route admixed with PD solutions. This mode of

administration is used for either local effects, such as in the treatment of peritonitis, or distant effects where the i.p. route is used for convenience.

The three main therapeutic additives to PD solutions were examined for their modulating effect on antibiotic activity: 1) standard heparin (Heparin Leo), 2) insulin (Humulin R, Lilly, Indianapolis, U.S.A), and 3) urokinase (Abbott Laboratories, Montreal, Canada). A new form of low-molecular-weight heparin, (Enoxaparin, Lovenox, Rhone-Poulenc Rorer Canada) which is still under clinical trial was also examined for its modulating effect on rifampin activity. Heparin is administered to patients, ranging in concentrations from 500 to 1000 U/L, to prevent clot formation and occlusion of the indwelling catheter. Approximately a third of CAPD patients are diabetics and are given insulin, in various concentrations, through the i.p. route. Urokinase is administered to patients, ranging in concentrations from 500 to 250,000 U/L, in order to promote the lysis of clots formed within the catheter lumen. The above mentioned therapeutic drug additives were tested in standard laboratory medium (peptone water) and in PD fluids, both fresh solution and spent fluid.

6. Morphological Assessment of Bacterial Biofilms by Scanning Confocal Laser Microscopy

The morphological assessment of experimental S. epidermidis biofilms in the fully hydrated state was conducted using a scanning confocal laser microscope (SCLM, Leica Canada). Two experimental situations were chosen for study: the control situation in the standard laboratory culture medium peptone water and the test situation following exposure to the antibiotic gentamicin (10 μg/ml) for 24 hours.

SCLM is a form of ultra-violet microscopy in which the digitized images are processed by computer-generated algorithms to permit increased

resolution, reduction of haze resulting from scatter and diffraction, optical slicing and the construction of 3-D images. Of particular value is the ability to examine fully hydrated specimens without dehydration-induced distortion or artifacts produced by cell death.

The bacterial biofilms were prepared for SCLM analysis by labelling with buffered fluorescein isothiocyanate (FITC, 389.4 MW, Sigma, No-R9379, St. Louis, MO). Buffering of the FITC was achieved under sterile conditions according to the following 3 steps: 1) Two separate stock buffer solutions were prepared, a 0.5 molar Na_2CO_3 buffer (pH 9.6) and a 0.01 molar K_2HPO_4 buffer (pH 7.2); 2) A working solution was prepared by adding 1.25 ml of 0.5 molar Na_2CO_3 buffer (pH 9.6) to 5.5 ml of 0.01 molar K_2HPO_4 buffer (pH 7.2) and 5.5 ml of a 0.85% saline solution; and 3) 5 mg of FITC was added to the buffered saline working solution which was then filtered with an inline syringe filter (0.22 μm) to make up a buffered FITC stock solution stored at 4°C.

Under sterile conditions the biofilms were rinsed in distilled water and submerged in five ml of the FITC stock solution for 5 to 15 min to allow ample time for the FITC to permeate the biofilm. Following a rinse in distilled water to remove excess FITC, the biofilm was covered with a coverslip and immediately examined by SCLM.

Images were obtained using the SCLM located in the Center for Research in Neuroscience at the Montreal General Hospital, equipped with a 100 X 1.3-numerical aperture oil immersion and a 40 X 1.32-numerical aperture oil immersion. The SCLM was also equipped with an argon laser with emission lines at 488 nm and 514 nm. The FITC was excited with the excitation emission line of 488 nm and long pass barrier filter OG 515 nm. Beam scanning through sequential increments (1 μm) horizontal optical sections of the biofilm preparations was facilitated through the use of galvinometrically controlled

mirrors. Images were converted to an analog video signal and displayed on a standard color monitor. Images were selected on the basis of optimum resolution and printed using a standard black and white video printer (Secocia).

Table 4a. Characteristics of rifampin-sensitive Staphylococcus epidermidis MGH #2503 (Vitek biotype # 77466044010)

Peptone base	+	Optochin	+	Tetrazolium red	+
40% Bile	+	Argenine	-	Salicin	-
Dextrose	+	Mannitol	-	Pullulan	-
Sucrose	+	Arabinose	-	Xylose	-
Melibiose	-	Cellobiose	-	10% Bile	+
Coagulase	-	Hemicellulase	+	Novobiocin	-
Bacitracin	+	Urea	+	Sorbitol	-
Esculin	-	Raffinose	-	Inulin	-
Lactose	+	Pyruvate	+	Catalase	+
Trehalose	-	Ribose	-		
Melezitose	-	6% NaCl	+		

Table 4b. Characteristics of rifampin-resistant Staphylococcus epidermidis #2503RR (Vitek biotype # 77464044010)

Peptone base	+	Optochin	+	Tetrazolium red	+
40% Bile	+	Argenine	-	Salicin	-
Dextrose	+	Mannitol	-	Pullulan	-
Sucrose	+	Arabinose	-	Xylose	-
Melibiose	-	Cellobiose	-	10% Bile	+
Coagulase	-	Hemicellulase	+	Novobiocin	-
Bacitracin	+	Urea	+	Sorbitol	-
Esculin	-	Raffinose	-	Inulin	-
Lactose	-	Pyruvate	+	Catalase	+
Trehalose	-	Ribose	-		
Melezitose	-	6% NaCl	+		

Table 5. Concentration/kinetics of antimicrobial activity of selected antibiotics against Staphylococcus epidermidis biofilms: Expression of results.

Antibiotics	µg/ml	Semi-quantitative assessment of residual biofilm metabolic activity[a] Days of exposure 1	2	3	4	5	Expression of results[b]
Rifampin	5	±	3+	3+	3+	3+	>5*
	10	-*	3+	3+	3+	3+	>5*
	20	-*	3+	3+	3+	3+	>5*
Cefazolin	5	3+	3+	3+	2+	1+	>5
	10	3+	3+	3+	1+	-	5
	20	3+	3+	2+	-	-	4
Vancomycin	5	3+	3+	3+	3+	2+	>5
	10	3+	2+	-	-	-	3
	20	2+	1+	-	-	-	3
Gentamicin	5	3+	2+	-	-	-	3
	10	2+	1+	-	-	-	3
	20	2+	-	-	-	-	2

[a]The antimicrobial activity of antibiotics in peptone water was estimated by measuring the residual metabolic activity of the bacterial biofilms after exposure, from 1-5 days, graded as:

1+ to 3+ for active, viable biofilms,
± occasional foci of active microcolonies
-* negative by visual inspection but culture positive
- complete kill, culture negative

[b]Results are expressed as number of days of exposure to the antibiotics required to produce cessation of metabolic activity.

*Rifampin-resistant survivors

Photographs of residual biofilm metabolic activity following exposure to these four antibiotics are presented in Figures 7-10.

Table 6. Test therapeutic agents

ANTIBIOTICS

Cell-wall active
- Cefazolin
- Cloxacillin
- Vancomycin

Aminoglycosides
- Amikacin
- Gentamicin
- Neomycin
- Netilmycin
- Streptomycin
- Tobramycin

Other
- Ciprofloxacin
- RIFAMPIN

PERITONEAL DIALYSIS SOLUTIONS

Fresh PD solutions in clinical use and under limited evaluation
Spent PD fluid (pooled effluent from MGH CAPD patients)

NON-ANTIBIOTIC THERAPEUTIC ADDITIVES TO PERITONEAL DIALYSIS SOLUTIONS

Heparin (standard and low molecular weight formulations)
Insulin
Urokinase

BACTERIAL BIOFILM ASSAY

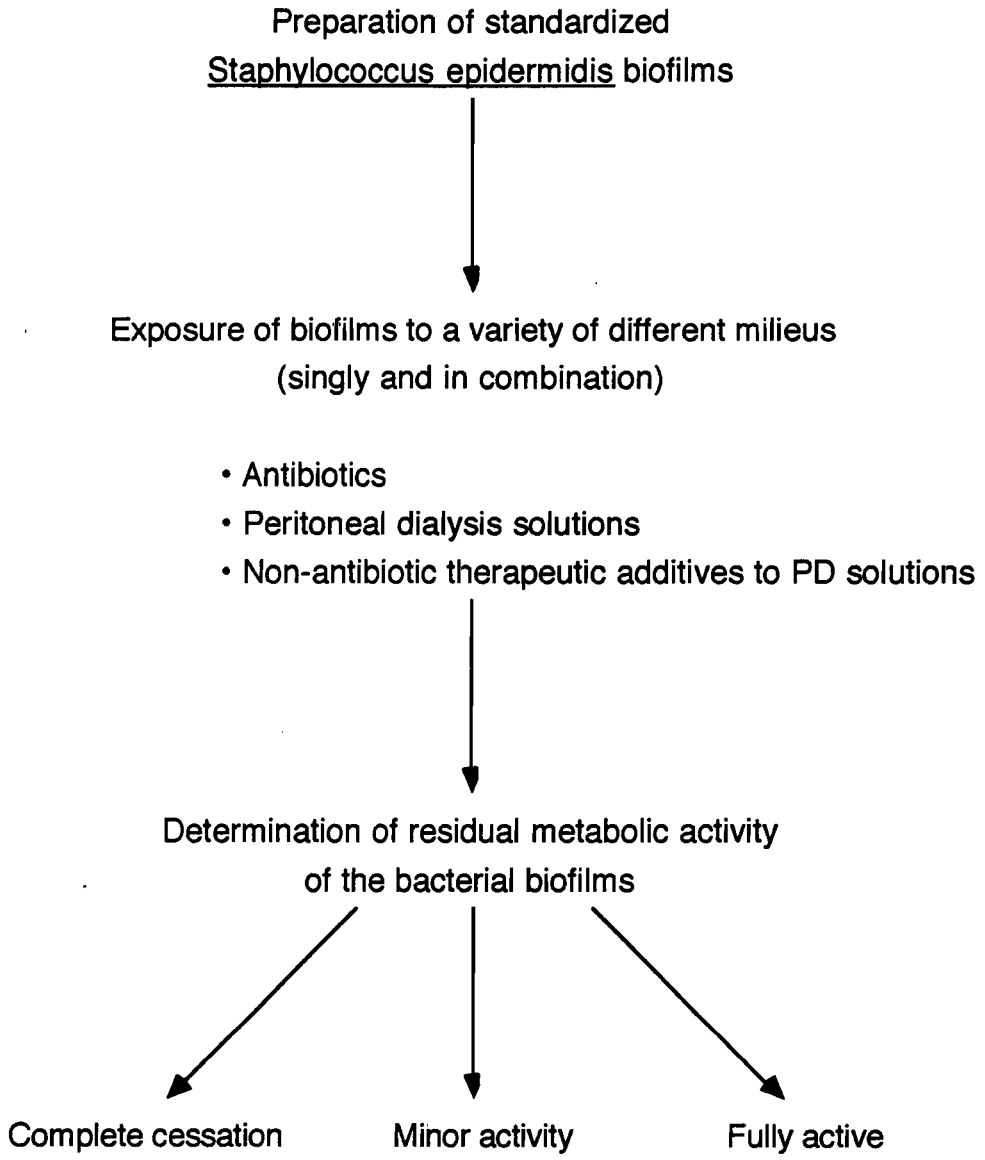


Figure 4. The key steps of the bacterial biofilm assay.

Figure 5. Illustrative steps of the bacterial biofilm assay.

a. Petri dish containing glass strips in a culture medium (TSB) inoculated with S. epidermidis in a log phase of growth. Biofilm formation will occur on the strips during overnight incubation at 37°C.

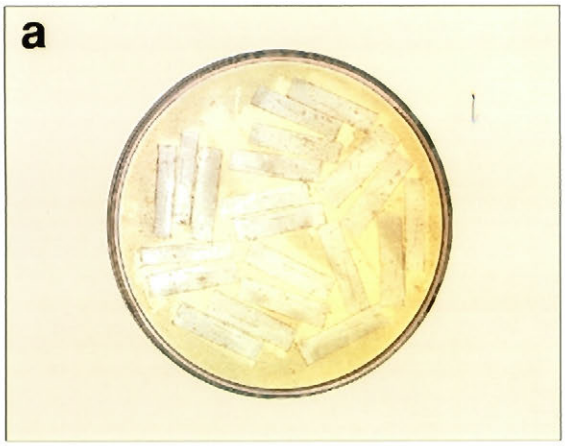
b. Comparison of the visual appearance of a glass strip coated with a S. epidermidis biofilm on the left and a plain glass strip on the right. Notice the opaque ground glass appearance of the biofilm. The letter "F" is engraved on the side of the glass free of biofilm.

c. Biofilm-coated glass strip immediately before placement into a screw-capped vial containing a selected milieu.

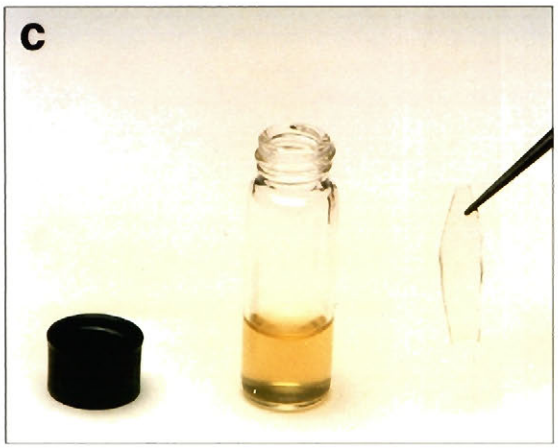
d. Close-up of a screw-capped vial containing a biofilm slide at the beginning of the incubation period. Daily replacement of test milieus with a sterile pasteur pipette was performed in order to avoid variations due to exhaustion of nutrients or pH effects.

e. Arrangement of screw-capped vials containing biofilms in selected milieus on racks ready for incubation at 37°C. Placement of the screw-capped vials in horizontal position provides optimal air/fluid ratio for bacterial metabolism.

f. Petri dish containing 5 biofilm strips placed onto the surface of the TTC agar with the biofilm side facing down and ready for further incubation at 37°C. The presence of metabolically active (viable) biofilm bacteria will be indicated by the development of a red pigmentation (formazan formation from the reduced TTC) within the biofilm.


a


b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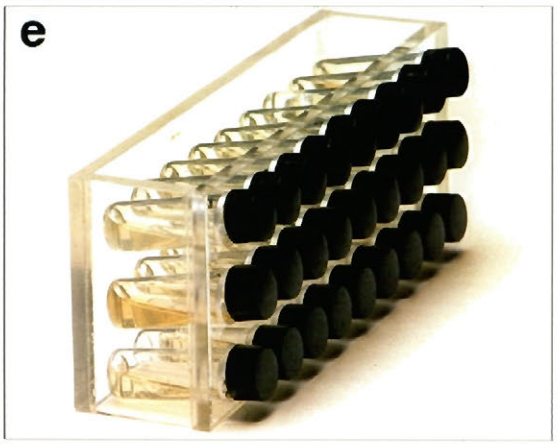
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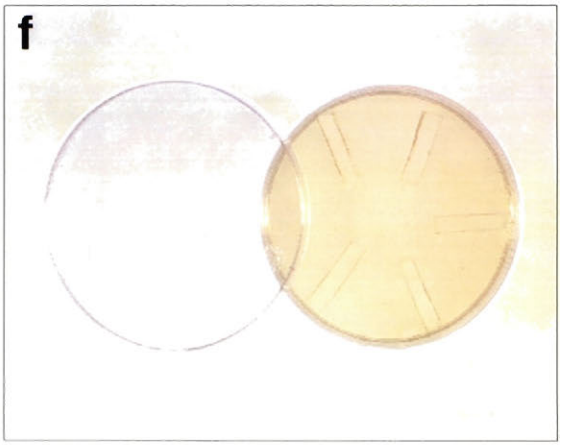

f

Figure 6. Determination of the residual metabolic activity of standardized S. epidermidis biofilms by the TTC method.

Close-up photograph of a petri dish containing five biofilm strips after 24 hours incubation at 37°C on the surface of the TTC agar.

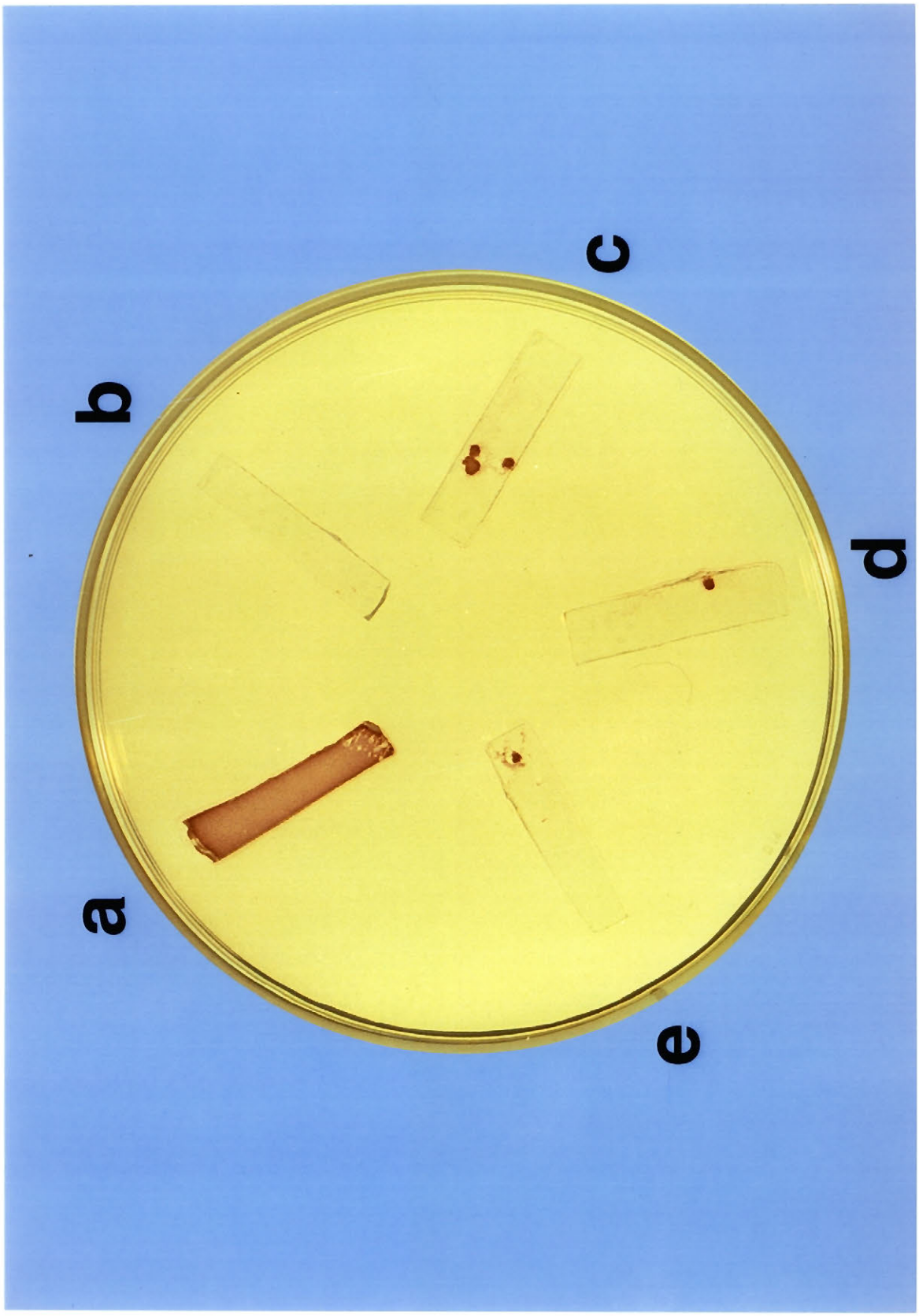
The determination of the residual biofilm metabolic activity on a TTC plate is determined by the visual assessment of the presence or absence of development of the red pigmentation (formazan production following TTC reduction by oxidative metabolism) within the biofilm.

Illustrative examples of the visual appearance of standardized S. epidermidis biofilm preparations as determined by the TTC method at the end of the exposure period in:

a. 1% peptone water (the standard positive control of growth included in all assays): development of a dense red pigmentation indicating full metabolic activity of the biofilm.

b. 4% formaldehyde (standard negative control of biofilm growth included in all assays): absent color change indicating complete cessation of metabolic activity of the biofilm (i.e. biofilm death).

c-e. rifampin at 3 different concentrations (5, 10, 20 μg/ml): development of scanty areas of red pigmentation indicating minor foci of persistent metabolic activity, corresponding to rifampin-resistant survivors.


a
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RESULTS

Antimicrobial Activity of Antibiotics against S. epidermidis Biofilms

The antibiotics used commonly in the management of CAPD peritonitis were examined for their antimicrobial activity against experimental S. epidermidis biofilms as an in vitro analogue of the clinical situation.

The Montreal General Hospital standard peritonitis protocol

The standard protocol currently used at the Montreal General Hospital for CAPD patients on presentation with peritonitis calls for the addition of vancomycin (1 mg/ml) together with an aminoglycoside (tobramycin, 6 μg/ml) and heparin (0.5 U/ml) to 2 liters of fresh peritoneal dialysis solution (1.5% dextrose) which is left to dwell in the peritoneal cavity for 6 hours. This regime is followed by further exchanges with the same agents but at reduced concentrations until the results of the gram stain and/or culture are available. Following identification of gram positive organisms the regime is modified by stopping the aminoglycoside.

The results for a range of concentrations of these agents, singly and in combination directed against standardized S. epidermidis biofilms exposed in a PD solution environment are presented in Table 7. The regime of vancomycin, tobramycin and heparin used on initial clinical presentation required 2 days for the complete kill of the bacterial biofilms which was the same duration required for vancomycin alone. Tobramycin alone had demonstrable lesser activity than the combination while heparin, which is not an antibiotic, showed no activity.

The four main antibiotics

This investigation focused on the antimicrobial activity against S. epidermidis biofilms of the four antibiotics rifampin, cefazolin, vancomycin

and gentamicin. The selection of these antibiotics was based primarily on clinical grounds since all four antibiotics are currently used in various combinations in various centers for the management of CAPD peritonitis and implant-associated infections in general. Rifampin was especially included because of its previously demonstrated singular action against experimental S. epidermidis biofilms (50, 107, 108, 133).

The results of an initial comparative study of the effects of concentration on the rate of antibiotic action (concentration/kinetics) of these four antibiotics are presented in Table 8. Rifampin possessed significant early bactericidal action against the biofilms even at the lowest dose of 5 μg/ml, however, the effect of rifampin was seen to be incomplete and rifampin-resistant foci readily repopulated the biofilm during the first 24 hours even in the presence of rifampin at increased concentrations (i.e. 20 μg/ml). Cefazolin, vancomycin and gentamicin used singly showed increasing activity (decreasing periods of exposure necessary to inhibit metabolic activity) with increasing concentrations of agent. Gentamicin produced the fastest killing of the biofilms (2 days at the higher concentration of 20 μg/ml). Photographs of the TTC results of this baseline experiment with the four antibiotics are presented in Figures 7-10.

Expanded range of antibiotics

The determination of the concentration/kinetics of antibiotic activity against the S. epidermidis biofilms was expanded to a larger panel of antibiotics by the addition of cloxacillin, streptomycin, ciprofloxacin, amikacin, netilmycin, neomycin and tobramycin (Table 9a). Each antibiotic was tested singly in peptone water to determine the necessary duration of exposure (measured in days) to produce cessation of metabolic activity in the standardized biofilms. Concentrations of 2.5, 5, 10 and 20 μg/ml were tested for each antibiotic.

In each situation, except for tobramycin which required more than 5 days (exceeding the maximal duration of test) to demonstrate an outcome, the rate of action was directly related to the concentration of antibiotic used. Three antibiotics (streptomycin, ciprofloxacin and vancomycin) showed a sharp increase of activity as the concentration was increased from 10 to 20 μg/ml. Of the 11 antibiotics studied in a single comparative batch (44 estimates per batch) streptomycin was notable for its speed of action compared to the other aminoglycosides.

Antibiotic combinations with rifampin

The investigation of a broad range of antibiotics combined with rifampin was conducted in order to determine those antibiotics which would complete rifampin action (Table 9b).

Among the cell-wall active class, cloxacillin and cefazolin acted synergistically with rifampin. Vancomycin (and ciprofloxacin) were less synergistic with rifampin but demonstrated dose-dependence. Among the aminoglycoside class there were marked differences in their interaction with rifampin. Amikacin and tobramycin had very little effect on rifampin action. Neomycin, netilmycin and gentamicin however demonstrated a clear antagonism with rifampin. The increasing antagonism with increasing dose was strikingly demonstrated by gentamicin. Streptomycin stood out from the other members of the aminoglycoside class by exerting marked synergy with rifampin which led to a complete and rapid kill of the biofilm. Three antibiotics (cloxacillin, cefazolin, streptomycin) in combination with rifampin produced complete killing within the shortest test exposure of one day.

Checkerboard analysis of aminoglycoside combination with rifampin

The disparate results of gentamicin and streptomycin when each were combined with rifampin were further explored using a checkerboard technique

(Table 10). This approach plotted time of exposure required to kill against concentration of antibiotics, alone and in combination.

A concentration-response relationship was shown when streptomycin was used alone. In combination with rifampin bactericidal synergy produced complete kill of the biofilm at 24 hours, even at the lowest concentration of 1.25 μg/ml for either antibiotic.

Gentamicin alone also showed a concentration-response relationship, but the activity of the rifampin-gentamicin combination was progressively antagonized as the concentration of gentamicin increased.

Triple antibiotic combinations with rifampin

Multi-drug regimes are part of the current initial management of implant-associated infection, therefore, the action of triple antibiotic combinations containing rifampin was also examined (Table 11). This multiple concentration/kinetic study assessed the effect of increasing the concentration of cefazolin, vancomycin and gentamicin when each in turn was added to rifampin in paired combinations with the other two agents.

The first column gives the duration of exposure of the rifampin combination pairs required to achieve a successful cidal outcome. The rifampin-cefazolin combination required one day; the rifampin-vancomycin combination 4 days; while the rifampin-gentamicin combination exceeded the test period of 5 days.

The first row of data indicates the effect of adding an antibiotic to the rifampin-cefazolin combination. The addition of vancomycin to the rifampin-cefazolin combination did not influence the efficacy of this desirable combination, which was already effective within the shortest exposure period of 24 hours. However the addition of gentamicin impaired the efficacy of the

combination and was concentration-related increasing the required duration of exposure progressively from 3 to 5 days.

The second row of data examines the addition of an antibiotic to the rifampin-vancomycin combination. The addition of cefazolin at even the lowest dose (2.5 μg/ml) to the rifampin-vancomycin combination improved activity to equal the efficacy of the rifampin-cefazolin combination as shown in the first column. The addition of gentamicin antagonized fully the efficacy of rifampin whether it was alone (column 1) or in combination with vancomycin (row 2).

The third row of data assessing the effect of adding an antibiotic to the rifampin-gentamicin combination demonstrated that the mutual antagonism of the rifampin-gentamicin combination was improved by increasing concentrations of cefazolin (to the required duration of 4 days) but unaffected by the addition of vancomycin.

In these triple combinations the data indicate the superiority of the rifampin-cefazolin combination, the minimal contribution of vancomycin, the significant and progressive antagonism of rifampin activity by gentamicin.

The effect of antibiotics on rifampin-resistant S. epidermidis biofilms

The efficacy of the four main antibiotics (rifampin, cefazolin, vancomycin and gentamicin) against rifampin-sensitive S. epidermidis biofilms (MGH #2503) was compared with their efficacy directed against biofilms prepared with a rifampin-resistant S. epidermidis strain (#2503RR) (Table 12). The striking ease of rifampin in negotiating the biofilm matrix barrier had led to speculation whether this facility would be retained in biofilms formed by rifampin-resistant bacteria, being detectable by the continuing demonstration of synergistic and antagonistic interactions.

The rapid but incomplete effect of rifampin when used alone on the rifampin-sensitive biofilms was not observed against the rifampin-resistant ones.

More importantly the antibiotics synergistic with rifampin (cefazolin and vancomycin) against the rifampin-sensitive biofilms lost their synergy against the rifampin-resistant biofilms. The latter observation is of fundamental importance since a prime action of rifampin had been suggested to affect biofilm matrix permeability directly, thereby permitting other antibiotics more rapid and direct access to the enclosed bacteria. The absence of cefazolin or vancomycin accelerated activity against these rifampin-exposed resistant biofilms confirms the absence of direct rifampin action on the biofilm matrix. The rapid action of rifampin on rifampin-sensitive S. epidermidis biofilms, therefore, results from its direct action on the enclosed susceptible bacteria alone.

The antagonism of rifampin with gentamicin was still noted implying incompatibility in the biofilm environment or a form of exclusion at the biofilm surface. The most rapid results (killing) of the rifampin-resistant biofilms were no longer obtained by the rifampin-cefazolin combination as in rifampin-sensitive biofilms but now by vancomycin alone.

Morphological Assessment of S. epidermidis Biofilms.

Using scanning confocal laser microscopy (SCLM), the morphology of experimental S. epidermidis biofilms was examined to determine the appearance of both cocci and the matrix and their structural relationship within fully hydrated biofilms.

In preliminary experiments both the bacteria and the matrix of biofilms were shown to directly differentially bind fluorescein isothiocyanate, thus

simplifying the procedure considerably. The bacteria fluoresced brightly, while the biofilm matrix was perceived as a separate weakly fluorescent zone clearly distinct from the black background.

The morphological studies were performed on biofilms exposed for 24 hours to two environmental extremes: the control environment of peptone water, sustaining metabolic activity of the biofilm, and after exposure to the antibiotic gentamicin (10 μg/ml) which will lead to cessation of metabolic activity after 2 days (Table 8). Representative images of the SCLM assessment on S. epidermidis biofilms are presented in Figures 11 and 12. These historically are the first reported SCLM images of S. epidermidis biofilms.

The microphotographs of Figure 11 present the SCLM appearance of a fully viable biofilm showing clusters of cocci enclosed in an amorphous extracellular matrix. The main findings are the easily identifiable bacteria in close proximity to the continuous dense surrounding matrix.

The microphotographs of Figure 12 present the SCLM appearance of a gentamicin-treated biofilm showing striking widespread disorganization of the bacterial biofilm. Marked structural alterations of both the bacterial population and the extracellular matrix are seen.

Biochemical Characterization of Peritoneal Dialysis Solutions

Table 13 presents the main characteristics provided by the manufacturers of the peritoneal dialysis solutions clinically used in 1994, some being commercially available worldwide (dextrose, amino acids) while others are undergoing large clinical trials (dextrose/bicarbonate and polyglucose) or are in limited use in selected dialysis units (glycerol). The chemical composition of the PD solutions differed in many respects, principally in the

nature of the osmotic agent (dextrose, amino acids, glycerol and polyglucose) and of the buffer (lactate, bicarbonate). Additional differences were related to electrolyte concentrations such as calcium. These significant differences in the constitution of the PD solutions were accompanied by correspondingly large differences in osmolarity (ranging from 272 to 410 mOsm/L) and in pH (ranging from 5.2 to 7.0).

The results of the biochemical tests performed by autoanalyzer on a representative fresh PD solution (4.25% dextrose, lactate buffer, Ca^{++} 1.62 mmol/L), individual and pooled spent fluids are presented in Tables 14 and 15. Table 14 describes the differences in constitution between fresh PD solution, pooled spent PD fluid and peptone water (as a control medium). When spent fluid was recovered from the ten stable CAPD patients and analyzed biochemically, the recovered fluid displayed three measurable differences from fresh PD solution: 1) the presence of several constituents absent in fresh solutions (bicarbonate, urea, creatinine, protein, albumin, phosphate); 2) increase in concentration of several constituents (potassium, chloride, uric acid); and 3) fall in concentration of others components (glucose, lactate, calcium). It is noteworthy that both PD fluids are different from the peptone water control medium particularly with respect to glucose, urea, calcium, magnesium and iron which are all considered critical elements to bacterial metabolism and growth.

Table 15 shows the variation between spent PD fluid samples from ten individual CAPD patients emphasizing the consistency in the characteristics of the spent fluid among each patient.

The Influence of Peritoneal Dialysis Solutions on Antibiotic Action against S. epidermidis Biofilms (rifampin-sensitive and rifampin-resistant)

Fresh PD solution

The modulating effects of six different PD solutions on the activity of antibiotics directed against rifampin-sensitive and rifampin-resistant S. epidermidis biofilms were assessed. Table 16a shows the efficacy of the antibiotics in the different PD solutions against rifampin-sensitive S. epidermidis biofilms. The PD solutions exerted a variable effect on antibiotic action. All the solutions acted synergistically with rifampin, accelerating and completing its bactericidal action within a 24 hours exposure period.

Dextrose with bicarbonate and polyglucose accelerated cefazolin, vancomycin and gentamicin activity compared to dextrose (Ca^{++} 1.25 and 1.62 mmol/L) and glycerol. The amino-acid solution showed a similar beneficial effect on vancomycin and gentamicin, but did not improve cefazolin activity as did the dextrose-bicarbonate and polyglucose solution.

The dextrose (Ca^{++} 1.25 mmol/L) solution fared badly in this test of synergistic activity but all six PD solutions neutralized in varying degree the rifampin-gentamicin antagonism. Across the board, dextrose-bicarbonate and polyglucose PD solutions demonstrated the most constant and favorable outcomes.

Table 16b demonstrated the efficacy of the antibiotics in the different PD solutions against rifampin-resistant S. epidermidis biofilms. Once again, all the PD solutions exerted a comparable effect on the antimicrobial activity of the antibiotics. However, rifampin was ineffective against the biofilms during the five days of exposure in the presence of the different PD solutions with the exception of the dextrose-bicarbonate. It should be noted that the

dextrose-based bicarbonate solution as a milieu was synergistic with all the antibiotics, singly and in combination, bringing about a bactericidal outcome.

Spent PD fluid

Table 17 demonstrates the effect of modulating rifampin activity in an environment of PD solutions and spent fluids. The Table shows that in the control environment of peptone water, exposure of the standardized biofilm preparations to rifampin resulted in predominant killing in the first day, but by the second day the biofilms were repopulated by rifampin-resistant survivors.

Exposure of the biofilm preparations to rifampin in a fresh PD solution environment resulted in sterilization of the biofilm during the first day of exposure. Further incubation failed to demonstrate any surviving organisms.

Exposure of the biofilms to rifampin in a spent PD fluid environment resulted in predominant killing of the biofilms by the first day of exposure, but further incubation in an antibiotic-free neutral laboratory medium confirmed the presence of rifampin-resistant survivors. Exposure of the biofilm preparations to the rifampin-spent PD solution environment for two days however resulted in sterilization of the biofilms.

Varying the concentration of rifampin from 5 to 20 μg/ml produced no difference in the antibacterial outcomes: the variation in rifampin response was therefore attributed to the change of the environment of the antibiotic-biofilm interaction.

The Influence of Non-antibiotic Therapeutic Agents (common additives to PD solutions) on Rifampin Activity against S. epidermidis Biofilms (rifampin-sensitive and rifampin-resistant)

Non-antibiotic therapeutic agents (heparin, insulin and urokinase) commonly added to PD solutions were assessed for the potential to interact with rifampin activity directed against S. epidermidis biofilms (Table 18). This assessment was conducted using peptone water (for baseline comparison), fresh and spent PD solutions as vehicles of characterized composition, using both rifampin-sensitive and rifampin-resistant S. epidermidis biofilm preparations.

Rifampin-sensitive biofilms

The rifampin-sensitive biofilms when exposed to rifampin (10 μg/ml) for periods of 1 to 5 days in a peptone water milieu were not killed, but were repopulated by rifampin-resistant survivors. The 3 additives did not affect the outcome.

In contrast, in fresh PD solutions as an environment, the biofilms were killed within 24 hours of exposure and, again, the additives did not affect the outcome.

Rifampin in spent dialysis fluid killed the biofilms by the second day of exposure. A difference was noted between the three additives in that urokinase accelerated rifampin action by the first day.

An alternative form of heparin (low molecular weight heparin) was also tested but no difference in outcome from that obtained from standard heparin was seen.

Rifampin-resistant biofilms

There was a striking contrast in outcomes using rifampin-resistant biofilms. Rifampin produced no demonstrable effect on biofilm viability whether

whether exposed in an environment of peptone water, fresh or spent dialysis fluids, with or without the presence of additives. The modulating effect of the environment noted for rifampin-sensitive biofilms was lost.

Both the environmental changes and the susceptibility of the biofilm bacteria markedly influenced the outcome of exposure to rifampin, whereas the presence of non-antibiotic therapeutic additives did not.

Table 7. Effect of various concentrations[a] of clinically relevant agents[b] alone and in combination against Staphylococcus epidermidis biofilms.

	Agents			Antimicrobial activity in	
	Vancomycin (mg/ml)	Tobramycin (μg/ml)	Heparin (U/ml)	Peptone water control	PD solution (1.5% dextrose)
1. On clinical presentation	0.1	0.6	0.5	>5	2
	1.0	**6**	**0.5**	**5**	**2**
	10	60	0.5	>5	4
2. Following identification of gram positive organisms	0.1	-	0.5	>5	3
	1.0	**-**	0.5	**>5**	**3**
	10	-	0.5	>5	3
3. Agents alone	0.1	-	-	5	3
	1.0	-	-	5	2
	10	-	-	5	2
	-	0.6	-	>5	>5
	-	6	-	>5	5
	-	60	-	>5	3
	-	-	0.5	>5	>5

[a]Highlighted concentrations are those used clinically.
[b]Agents used in the standard protocol of treatment of peritonitis in CAPD patients at the Montreal General Hospital. Vancomycin, tobramycin and heparin are used on clinical presentation. Vancomycin and heparin are used upon identification of gram positive organisms from the peritoneal fluid.
Results are expressed as the number of days of exposure to the antibiotics required to produce cessation of metabolic activity.

Table 8. The relationship of antibiotic concentration and rate of kill of Staphylococcus epidermidis biofilms.

	Antibiotic concentration (μg/ml)		
	5	10	20
Rifampin	>5*	>5*	>5*
Cefazolin	>5	5	4
Vancomycin	>5	3	3
Gentamicin	4	3	2
Controls			
Peptone water (1%)	>5		
Formaldehyde (4%)	1		

*Rifampin-resistant survivors
Assay conducted in the control environment of 1% peptone water.
Results are expressed as the number of days of exposure to the antibiotics required to produce cessation of metabolic activity.

Table 9a. Antimicrobial activity against Staphylococcus epidermidis biofilms: The effect of concentration on single antibiotics.

	Antibiotic concentration (μg/ml)			
	2.5	5	10	20
Rifampin	>5*	>5*	>5*	>5*
Streptomycin	>5	>5	5	2
Ciprofloxacin	>5	5	5	3
Vancomycin	>5	>5	>5	3
Gentamicin	4	3	3	3
Cefazolin	>5	>5	4	4
Netilmycin	5	5	4	4
Neomycin	>5	5	4	4
Cloxacillin	>5	>5	5	5
Amikacin	>5	>5	5	5
Tobramycin	>5	>5	>5	>5

*Rifampin-resistant survivors
Assay conducted in the control environment of 1% peptone water.
Results are expressed as the number of days of exposure to the antibiotics required to produce cessation of metabolic activity.
Note the Table is ordered by the duration required to achieve cessation of activity at the highest concentration of 20 μg/ml.

Table 9b. Antimicrobial activity against Staphylococcus epidermidis biofilms: The effect of concentration on combinations of antibiotics with rifampin.

	Antibiotic concentration (μg/ml) (in combination with rifampin 10 μg/ml)			
	2.5	5	10	20
Cloxacillin	1	1	1	1
Cefazolin	1	1	1	1
Streptomycin	1	1	1	1
Vancomycin	>5	5	2	1
Ciprofloxacin	3	3	3	2
Amikacin	5	5	5	5
Gentamicin	4	4	5	>5
Netilmycin	>5	>5	>5	>5
Neomycin	>5	>5	>5	>5
Tobramycin	>5	>5	>5	>5
Rifampin alone	>5*	>5*	>5*	>5*

*Rifampin-resistant survivors
Assay conducted in the control environment of 1% peptone water.
Results are expressed as the number of days of exposure to the antibiotics required to produce cessation of metabolic activity.
Note the Table is ordered by the duration required to achieve cessation of activity at the highest concentration of 20 μg/ml.

Table 10. Antimicrobial activity of streptomycin and gentamicin against Staphylococcus epidermidis biofilms: Effect of various concentrations of both agents, alone and in combination with rifampin (checkerboard technique).

		Agent alone	Agent with rifampin (μg/ml)		
			1.25	10	40
Concentration (μg/ml)					
Rifampin	1.25	>5*			
	10	>5*			
	40	>5*			
Streptomycin	1.25	>5	1	1	1
	10	5	1	1	1
	40	3	1	1	1
Gentamicin	1.25	>5	3	3	3
	10	5	5	>5	5
	40	5	>5	>5	>5

*Rifampin-resistant survivors
Assay conducted in the control environment of 1% peptone water.
Results are expressed as the number of days of exposure to the antibiotics required to produce cessation of metabolic activity.

Table 11. Antimicrobial activity of antibiotic combinations with rifampin against Staphylococcus epidermidis biofilms: The effect of concentration on the activity of double and triple combinations.

Rifampin pair (10 μg/ml each)	Rifampin pair	Triple combinations (rifampin pair with:)											
		Cefazolin (μg/ml)				Vancomycin (μg/ml)				Gentamicin (μg/ml)			
		2.5	5	10	20	2.5	5	10	20	2.5	5	10	20
Rifampin + cefazolin	1					1	1	1	1	3	4	5	5
Rifampin + vancomycin	4	1	1	1	1					>5	>5	>5	>5
Rifampin + gentamicin	>5	>5	5	5	4	>5	>5	>5	>5				

*Rifampin-resistant survivors
Assay conducted in the control environment of 1% peptone water.
Results are expressed as the number of days of exposure to the antibiotics required to produce cessation of metabolic activity.

Table 12. The antimicrobial activity of antibiotics, singly and in combination with rifampin, against rifampin-sensitive and rifampin-resistant Staphylococcus epidermidis biofilms.

Antibiotics	Rifampin-sensitive S. epidermidis (MGH #2503)	Rifampin-resistant S. epidermidis (#2503RR)
Rifampin	>5*	>5
Cefazolin	>5	>5
Vancomycin	>5	5
Gentamicin	5	5
Rifampin + cefazolin	1	>5
Rifampin + vancomycin	1	5
Rifampin + gentamicin	>5	>5

*Emergence of rifampin-resistant survivors
Assay conducted in the control environment of 1% peptone water.
Results are expressed as the number of days of exposure to the antibiotics required to produce cessation of metabolic activity.
Each antibiotic was tested at a final concentration of 10 μg/ml.

Table 13. Differential characteristics of a panel of fresh peritoneal dialysis solutions.

PD solutions	Osmotic agent (g/L)	Osmolarity (mOsm/L)	pH	Buffer (mmol/L)	Na^+ (mmol/L)	Cl^- (mmol/L)	Ca^{++} (mmol/L)	Mg^{++} (mmol/L)
Dextrose[a]	Dextrose 15	345	5.2	Lactate 40	132	96	1.25	0.25
Dextrose[a]	Dextrose 15	347	5.2	Lactate 35	132	101.76	1.62	0.75
Dextrose/ bicarbonate[b]	Dextrose 15	361	7.0	Bicarbonate 34	134	104.5	1.75	0.5
Dextrin 20[c]	Glucose polymer 75	272	5.7	Lactate 40	133	97	1.75	0.25
Glycerol[d]	Glycerol 14	410	6.2	Lactate 35	132	102	1.75	0.75
Amino acids[e]	Amino acids 11.205	365	6.6	Lactate 40	132	105	1.25	0.25

All information supplied by the manufacturers:
[a]Baxter laboratories, JB9616, Toronto, Canada. [b]Bic 20,Fresenius, Germany
[c]Fresenius D-6830, Badhomburg, U.K. [d]Baxter 712-2, Castlebar, Ireland
[e]Baxter Laboratories, AX2009, Rome, Italy

Table 14. Biochemical characteristics of fresh and spent peritoneal dialysis fluids.

Parameters	Peptone water[a]	Fresh PD solution[b]	Pooled spent PD fluid[c]
pH	7.3	5.75	8.7
Osmolarity (mmol/Kg)	223	334	305
Glucose (mmol/L)	0	57.8	21.3
Sodium (mmol/L)	109	135	137
Potassium (mmol/L)	3	0.1	3.6
Bicarbonate (mmol/L)	0	0	19
Chloride (mmol/L)	92	96	108
Lactate (mmol/L)	0.51	15.78	2.3
Urea (mmol/L)	0.4	0	22.6
Creatinine (μmol/L)	213	0	803
Uric acid (μmol/L)	7	5	270
Total protein (g/L)	5	0	1
Albumin (g/L)	0	0	1
Calcium (mmol/L)	0.12	1.73	1.38
Phosphate (mmol/L)	0.9	0	1.21
Magnesium (mmol/L)	0.1	0.71	0.74
Iron (μmol/L)	11	0	0

Routine biochemistry testing was performed at the MGH Department of Medical Biochemistry

[a]1% peptone water

[b]Fresh PD solution (4.25% dextrose, lactate buffer, Ca^{++} 1.62 mmol/L)

[c]Pooled spent PD fluid (from 4.25% dextrose, other specifications as above) from ten MGH CAPD patients

Biochemical results from the ten individual spent fluids are given in Table 15.

Table 15. Biochemical characteristics of individual spent peritoneal dialysis fluid from ten CAPD patients.

Parameters	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10
pH	8.6	8.6	8.65	8.5	8.8	8.25	8.6	8.55	8.2	8.2
Osmolarity (mmol/Kg)	306	293	293	303	300	311	306	322	304	304
Glucose (mmol/L)	13.4	18.1	12.4	27.5	23.8	13.8	34.2	23.9	27.7	16
Sodium (mmol/L)	136	139	140	132	139	138	130	142	129	138
Potassium (mmol/L)	4.0	2.8	4	4.1	2.4	4.1	3.1	3.3	3.9	4.3
Bicarbonate (mmol/L)	20	21	20	17	20	20	19	19	17	21
Chloride (mmol/L)	109	110	114	104	109	110	101	110	99	110
Lactate (mmol/L)	2.12	2.08	1.33	2.57	2.51	1.19	2.08	3.14	3.93	1.89
Urea (mmol/L)	20.4	21.1	14.7	26.6	15.6	31.8	21.1	27.4	23.8	23.5
Creatinine (μmol/L)	697	760	501	815	736	892	522	1207	774	1106
Uric acid (μmol/L)	223	263	217	269	259	308	208	363	247	336
Total protein (g/L)	2	2	2	0	1	1	1	2	1	2
Albumin (g/L)	1	1	1	0	0	1	0	1	0	1
Calcium (mmol/L)	1.4	1.36	1.19	1.28	1.05	1.27	1.5	1.22	1.47	1.32
Phosphate (mmol/L)	1.1	1.21	1.27	1.22	0.76	1.66	0.9	1.26	1.02	1.41
Magnesium (mmol/L)	0.79	0.85	0.68	0.79	0.86	0.78	0.83	0.81	0.87	0.74
Iron (μmol/L)	0	0	0	0	0	0	0	0	0	0

Routine biochemistry testing of the spent fluids was performed at the MGH Department of Medical Biochemistry. Spent fluids were collected after overnight dwell with fresh PD solution (4.25% dextrose, lactate buffer, Ca^{++} 1.62 mmol/L) in ten stable MGH CAPD patients. The patients were free of peritonitis for the preceeding 6 months and not currently treated with any antibiotics or therapeutic additives.
Biochemical results for the pooled spent fluid from the ten CAPD patients are given in Table 14.

Table 16a. Modulating effect of peritoneal dialysis solutions on the antimicrobial activity of clinically relevant antibiotics, singly and in combination with rifampin, against rifampin-sensitive Staphylococcus epidermidis biofilms (MGH #2503).

Antibiotic (10 μg/ml)	Dextrose (Ca^{++} mmol/L)		Dextrose with bicarbonate	Amino acids	Poly glucose	Glycerol	Peptone water control
	1.25	1.62					
Rifampin	1	1	1	1	1	1	>5*
Cefazolin	>5	>5	4	>5	5	>5	>5
Vancomycin	>5	4	4	4	4	>5	>5
Gentamicin	5	>5	3	2	4	5	5
Rifampin + cefazolin	2	1	1	1	1	1	1
Rifampin + vancomycin	2	1	1	1	1	1	1
Rifampin + gentamicin	3	3	2	5	3	3	>5

*Emergence of rifampin-resistant survivors
Results are expressed as the number of days of exposure to the antibiotics required to produce cessation of metabolic activity.

Table 16b. Modulating effect of peritoneal dialysis solutions on the antimicrobial activity of clinically relevant antibiotics, singly and in combination with rifampin, against rifampin-resistant Staphylococcus epidermidis biofilms (#2503RR).

Antibiotic (10 μg/ml)	Dextrose (Ca^{++} mmol/L)		Dextrose with bicarbonate	Amino acids	Poly glucose	Glycerol	Peptone water control
	1.25	1.62					
Rifampin	>5	>5	4	>5	>5	>5	>5
Cefazolin	>5	>5	4	>5	>5	>5	>5
Vancomycin	>5	>5	4	5	>5	>5	5
Gentamicin	5	5	3	5	>5	5	5
Rifampin + cefazolin	>5	>5	4	>5	>5	>5	5
Rifampin + vancomycin	>5	>5	4	>5	>5	>5	5
Rifampin + gentamicin	>5	>5	5	>5	>5	>5	>5

Results are expressed as the number of days of exposure to the antibiotics required to produce cessation of metabolic activity.

Table 17. Influence of fresh and spent peritoneal dialysis fluids on the susceptibility of Staphylococcus epidermidis biofilms to rifampin.

Rifampin (μg/ml)	Peptone water control[a]			Fresh PD solution[b]			Pooled spent PD fluid[c]		
	5	10	20	5	10	20	5	10	20
Day 1	-*	-*	-*	-	-	-	-*	-*	-*
Day 2	+++	+++	+++	-	-	-	-	-	-
Day 3	+++	+++	+++	-	-	-	-	-	-
Day 4	+++	+++	+++	-	-	-	-	-	-
Day 5	+++	+++	+++	-	-	-	-	-	-

Routine biochemistry testing performed at the MGH Department of Medical Biochemistry.
[a]1% peptone water
[b]Fresh PD solution (4.25% dextrose, lactate buffer, Ca^{++} 1.62 mmol/L)
[c]Pooled spent PD fluid (from 4.25% dextrose, other specifications as above) from ten MGH CAPD patients
The effect of combining rifampin with PD fluids was estimated by measuring the residual metabolic activity of the bacterial biofilms after exposure, from 1-5 days, graded as:

+++ for active, viable biofilms,
-* for 99.9% initial kill,
- for complete kill.

Table 18a. Influence of non-antibiotic therapeutic additives to PD solutions on the susceptibility of rifampin-sensitive Staphylococcus epidermidis biofilms (MGH #2503) to rifampin (10 μg/ml).

	Peptone water control[a]				Fresh PD solution[b]				Pooled spent PD fluid[c]			
	NA	Hep	Ins	Uro	NA	Hep	Ins	Uro	NA	Hep	Ins	Uro
Day 1	-*	-*	-*	-*	-	-	-	-	-*	-*	-*	-
Day 2	+++	+++	+++	+++	-	-	-	-	-	-	-	-
Day 3	+++	+++	+++	+++	-	-	-	-	-	-	-	-
Day 4	+++	+++	+++	+++	-	-	-	-	-	-	-	-
Day 5	+++	+++	+++	+++	-	-	-	-	-	-	-	-

[a]1% peptone water
[b]Fresh PD solution (4.25% dextrose, lactate buffer, Ca^{++} 1.62 mmol/L)
[c]Pooled spent PD fluid (4.25% dextrose, other specifications as above) from ten MGH CAPD patients
Abbreviations: NA, No additives
Hep, Heparin,10 U/ml
Ins, Insulin,0.05 U/ml
Uro, Urokinase, 5 U/ml
The effect of combining rifampin with therapeutic additives in various milieus was estimated by measuring the residual metabolic activity of the bacterial biofilms after exposure, from 1-5 days, graded as:
+++ for active, viable biofilms,
-* for 99.9% initial kill,
- for complete kill.

Table 18b. Influence of non-antibiotic therapeutic additives to PD solutions on the susceptibility of rifampin-resistant Staphylococcus epidermidis biofilms (#2503RR) to rifampin (10 μg/ml).

	Peptone water control[a]				Fresh PD solution[b]				Pooled spent PD fluid[c]			
	NA	Hep	Ins	Uro	NA	Hep	Ins	Uro	NA	Hep	Ins	Uro
Day 1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Day 2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Day 3	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Day 4	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Day 5	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

[a]1% peptone water
[b]Fresh PD solution (4.25% dextrose, lactate buffer, Ca^{++} 1.62 mmol/L)
[c]Pooled spent PD fluid (from 4.25% dextrose, other specifications as above) from ten MGH CAPD patients
Abbreviations: NA, No additives
Hep, Heparin,10 U/ml
Ins, Insulin,0.05 U/ml
Uro, Urokinase, 5 U/ml
The effect of combining rifampin with therapeutic additives in various milieus was estimated by measuring the residual metabolic activity of the bacterial biofilms after exposure, from 1-5 days, graded as:
+++ for active, viable biofilms,
-* for 99.9% initial kill,
- for complete kill.

Figure 7. Concentration/kinetics of the antimicrobial activity of rifampin against Staphylococcus epidermidis biofilms.

The antimicrobial activity of rifampin against standardized S. epidermidis biofilms was determined over 5 days for three concentrations (5, 10, 20 μg/ml). The residual metabolic activity of the biofilms was determined following the selected exposure periods. For this purpose, the biofilm preparations were placed on TTC agar plates and incubated a further 24 hours at 37°C. The color development within the biofilm results from the formation of formazan (upon TTC reduction from oxidative metabolism) indicating viability of the biofilm bacteria. The permanent absence of color change indicates biofilm death. cessation of metabolic activity of the biofilms. The final reading of the results of this experiment is:

Rifampin, 5 μg/ml: >5*
10 μg/ml: >5*
20 μg/ml: >5*

*Rifampin-resistant survivors

Day 1- Transient absence of detectable biofilm metabolic activity at 10 and 20 μg/ml. Visible foci of rifampin resisters are present at 5 μg/ml.

Day 2- Repopulation of the biofilms by rifampin-resistant survivors is seen in the continuing presence of the 3 rifampin concentrations.

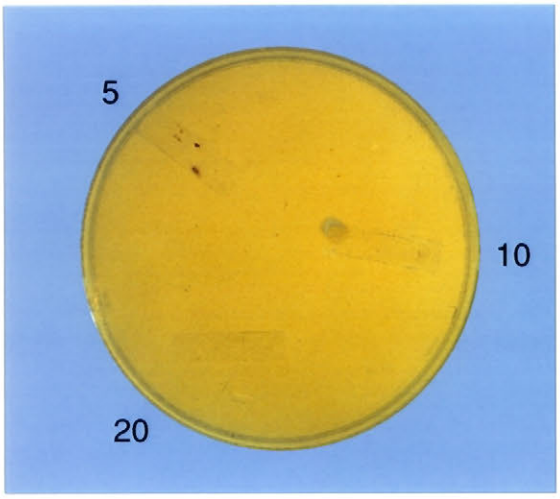
Day 3- Full metabolic activity of the rifampin-resistant biofilms.

Day 4- Full biofilm metabolic activity at all rifampin concentrations.

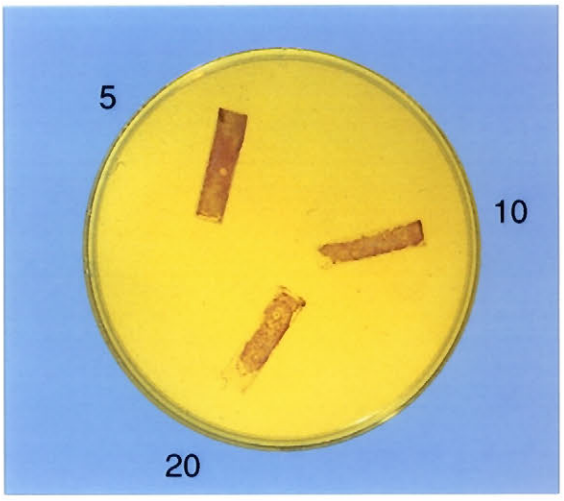
Day 5- Fully viable rifampin-resistant biofilms.

RIFAMPIN

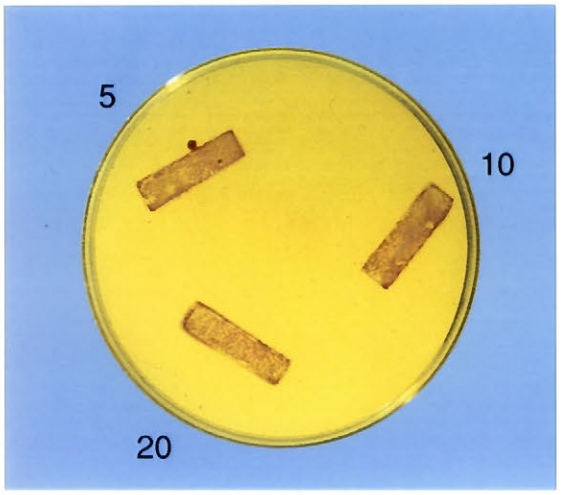
DAY 1



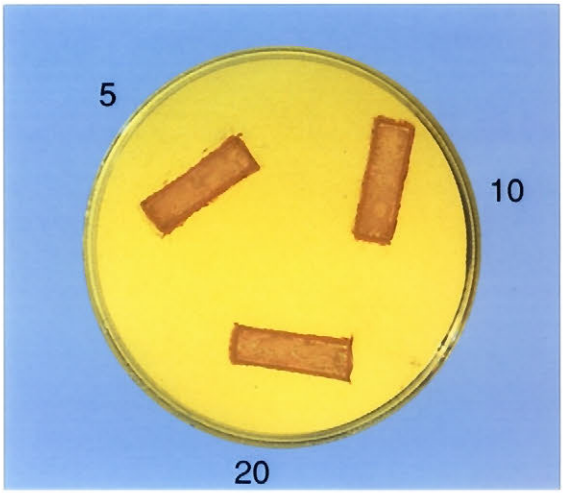
DAY 2



DAY 3



DAY 4



DAY 5

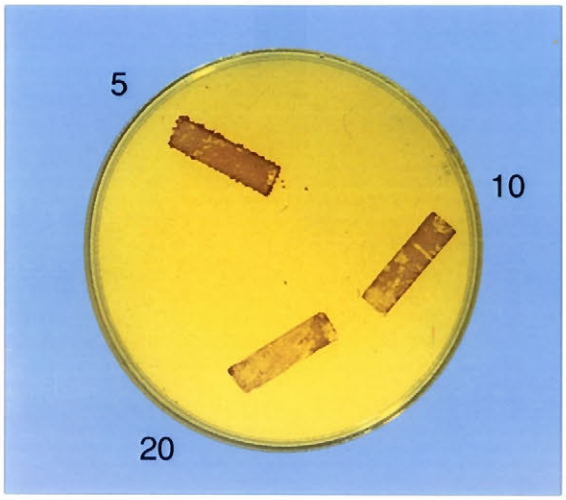


Figure 8. Concentration/kinetics of the antimicrobial activity of cefazolin against Staphylococcus epidermidis biofilms.

The antimicrobial activity of cefazolin against standardized S. epidermidis biofilms was determined over 5 days for three concentrations (5, 10, 20 μg/ml). The residual metabolic activity of the biofilms was determined following the selected exposure periods. For this purpose, the biofilm preparations were placed on TTC agar plates and incubated a further 24 hours at 37°C. The color development within the biofilm results from the formation of formazan (upon TTC reduction from oxidative metabolism) indicating viability of the biofilm bacteria. The permanent absence of color change indicates biofilm death. The results are expressed as the number of days required to produce cessation of metabolic activity of the biofilms. The final reading of the results of this experiment is:

Cefazolin, 5 μg/ml: >5
10 μg/ml: 5
20 μg/ml: 5

Day 1- Full metabolic activity of the biofilms is seen at the 3 test concentrations.

Day 2- No significant changes yet in the metabolic activity at the 3 test concentrations.

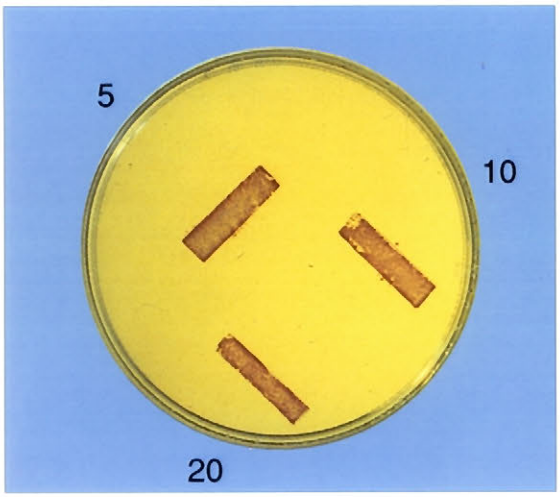
Day 3- A large area of absent metabolic activity is observed at 20 μg/ml.

Day 4- Marked loss of metabolic activity is seen at all test concentrations with only faint persistence of metabolic activity at the 10 and 20 μg/ml concentrations.

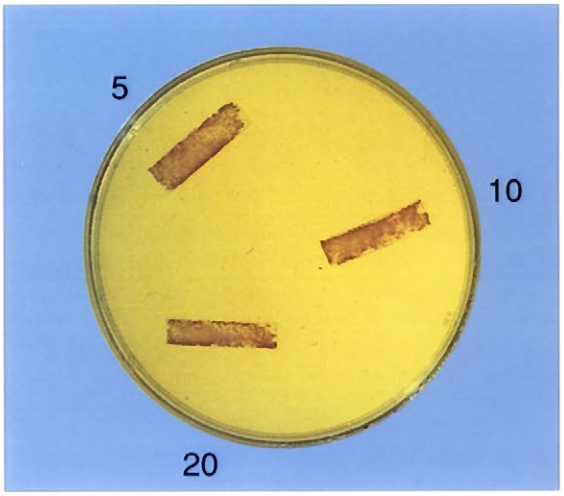
Day 5- Complete cessation of metabolic activity at 10 and 20 μg/ml. Faint persistence of metabolic activity is observed at 5 μg/ml.

CEFAZOLIN

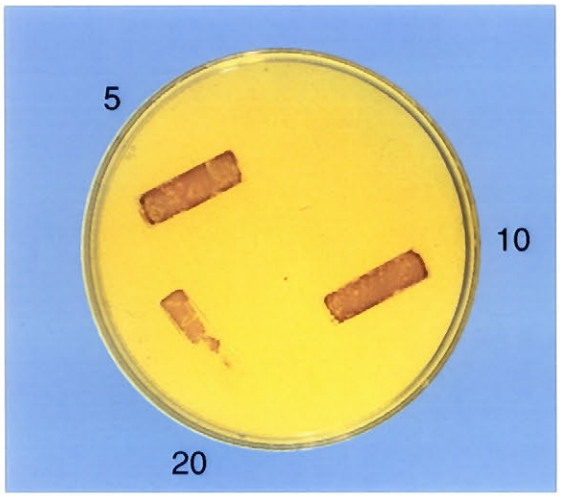
DAY 1



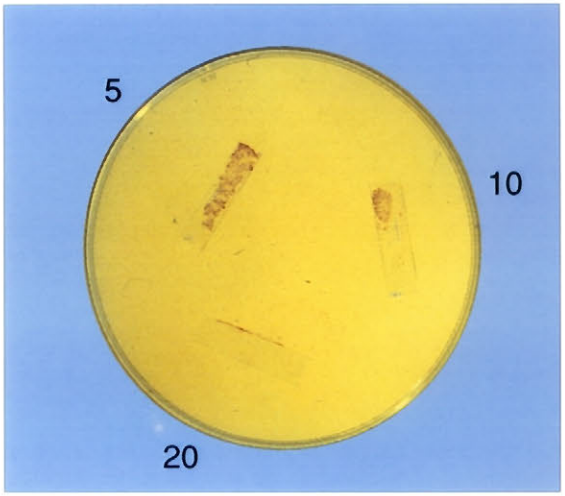
DAY 2



DAY 3



DAY 4



DAY 5

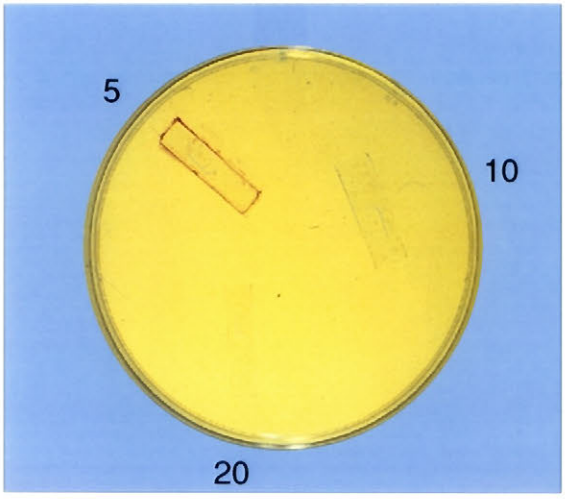


Figure 9. Concentration/kinetics of the antimicrobial activity of vancomycin against Staphylococcus epidermidis biofilms.

The antimicrobial activity of vancomycin against standardized S. epidermidis biofilms was determined over 5 days for three concentrations (5, 10, 20 μg/ml). The residual metabolic activity of the biofilms was determined following the selected exposure periods. For this purpose, the biofilm preparations were placed on TTC agar plates and incubated a further 24 hours at 37°C. The color development within the biofilm results from the formation of formazan (upon TTC reduction from oxidative metabolism) indicating viability of the biofilm bacteria. The permanent absence of color change indicates biofilm death. The results are expressed as the number of days required to produce cessation of metabolic activity of the biofilms. The final reading of the results of this experiment is:

Vancomycin,	5 μg/ml:	>5
	10 μg/ml:	3
	20 μg/ml:	3

Day 1- Metabolic activity is present in the biofilms at the 3 test concentrations. Patchy areas of absent metabolic activity are seen at the concentrations of 10 and 20 μg/ml concentrations.

Day 2- Further loss of metabolic activity is seen at the 20 μg/ml concentration.

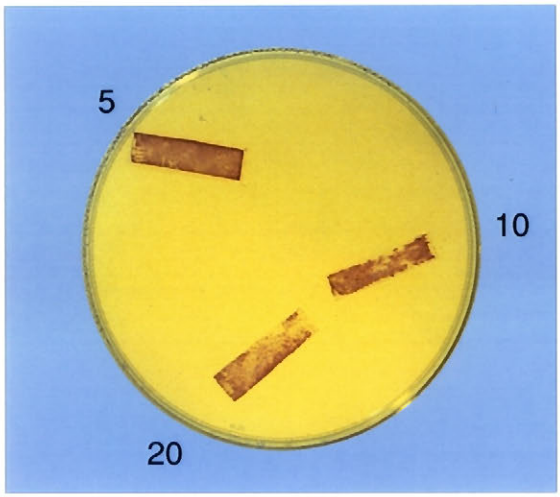
Day 3- Complete cessation of metabolic activity is observed at 10 and 20 μg/ml.

Day 4- Patchy areas of absent metabolic activity are now seen at 5 μg/ml.

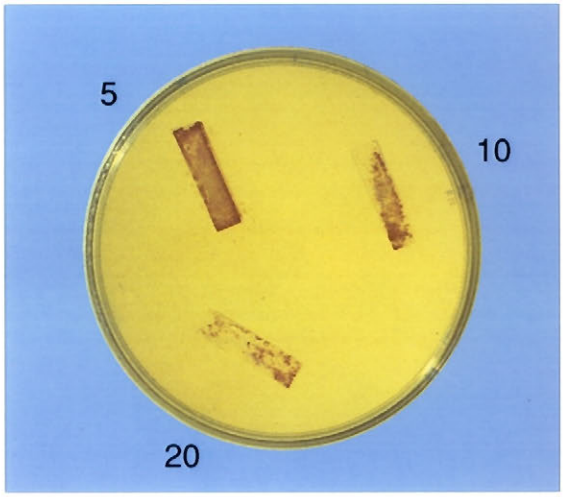
Day 5- Widespread destruction of the biofilm at 5 μg/ml with persistence of only slight metabolic activity.

VANCOMYCIN

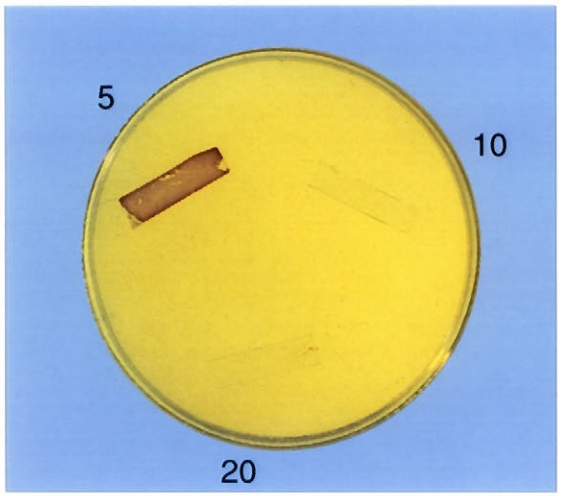
DAY 1



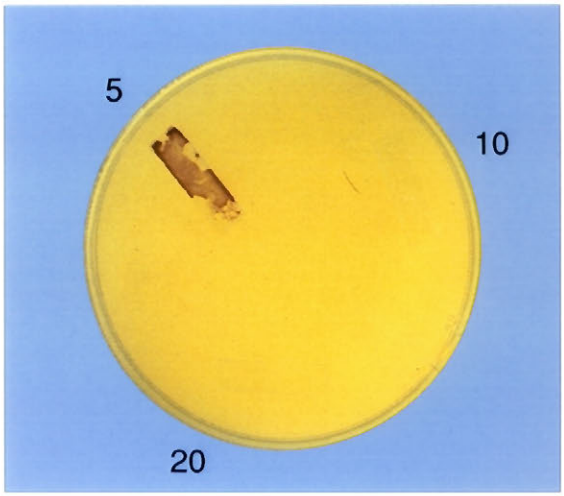
DAY 2



DAY 3



DAY 4



DAY 5

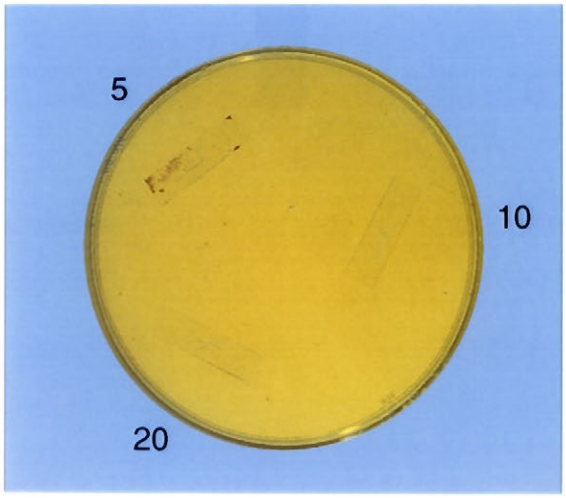


Figure 10. Concentration/kinetics of the antimicrobial activity of gentamicin against Staphylococcus epidermidis biofilms.

The antimicrobial activity of gentamicin against standardized S. epidermidis biofilms was determined over 5 days for three concentrations (5, 10, 20 μg/ml). The residual metabolic activity of the biofilms was determined following the selected exposure periods. For this purpose, the biofilm preparations were placed on TTC agar plates and incubated a further 24 hours at 37°C. The color development within the biofilm results from the formation of formazan (upon TTC reduction from oxidative metabolism) indicating viability of the biofilm bacteria. The permanent absence of color change indicates biofilm death. The results are expressed as the number of days required to produce cessation of metabolic activity of the biofilms. The final reading of the results of this experiment is:

Gentamicin, 5 μg/ml: 3
10 μg/ml: 3
20 μg/ml: 2

Day 1- Large confluent areas of absent metabolic activity are seen at 10 and 20 μg/ml and patchy areas of absent metabolic activity at 5 μg/ml.

Day 2- Complete cessation of metabolic activity at 20 μg/ml. Persistence of faint metabolic activity is observed at the 5 and 10 μg/ml concentrations.

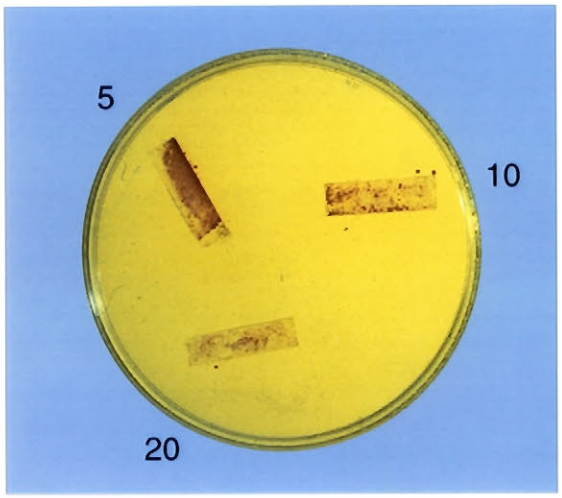
Day 3- Absent metabolic activity is seen at 10 and 20 μg/ml. Minimal metabolic activity is observed at the lowest test concentration of 5 μg/ml.

Day 4- Absence of biofilm metabolic activity at all concentrations.

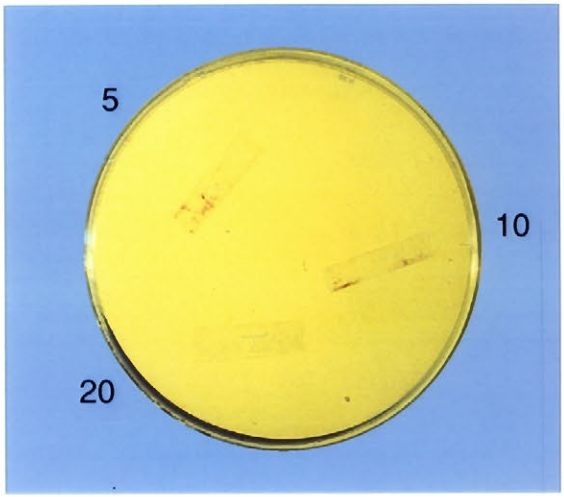
Day 5- Continued absence of biofilm metabolic activity at all gentamicin concentrations.

GENTAMICIN

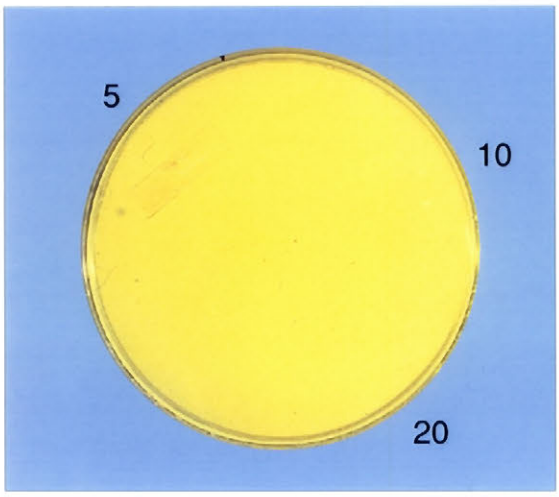
DAY 1



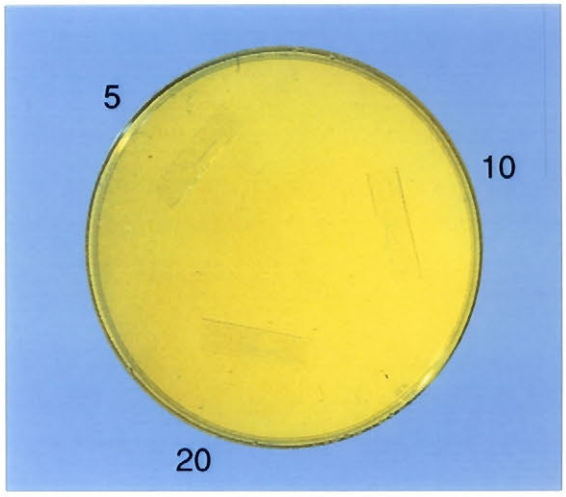
DAY 2



DAY 3



DAY 4



DAY 5

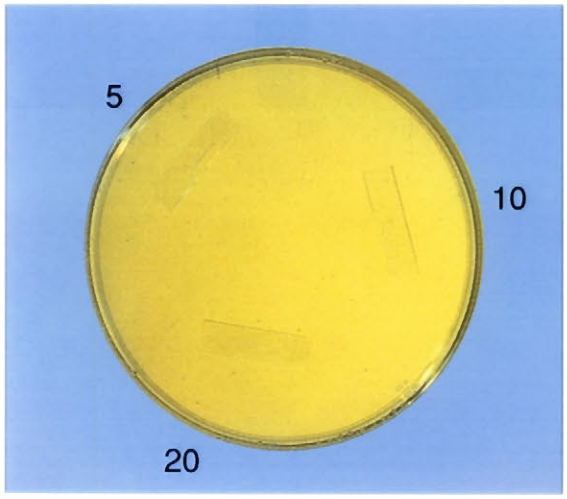


Figure 11a. Photomicrograph of an experimental Staphylococcus epidermidis biofilm (MGH #2503) on a glass surface in a control environment. Images obtained using a scanning confocal laser microscope (SCLM, magnification X 1050).

The biofilm was incubated for 24 hours in peptone water, a standard bacteriological culture medium. The biofilm was immediately prepared for the SCLM examination by short-term exposure to FITC, a fluorescent dye. The bacteria fluoresce brightly while the biofilm matrix is perceived as a separate low fluorescent zone clearly distinct from the black background.

Typical clusters of cocci are seen enclosed in an easily demonstrable extracellular matrix.

Notice the homogeneity of the bacterial population (well identifiable spherical bodies) and the smooth continuous border of the enclosing matrix. Notice also the distinct separation between the periphery of the clusters and the apposing side of the matrix.

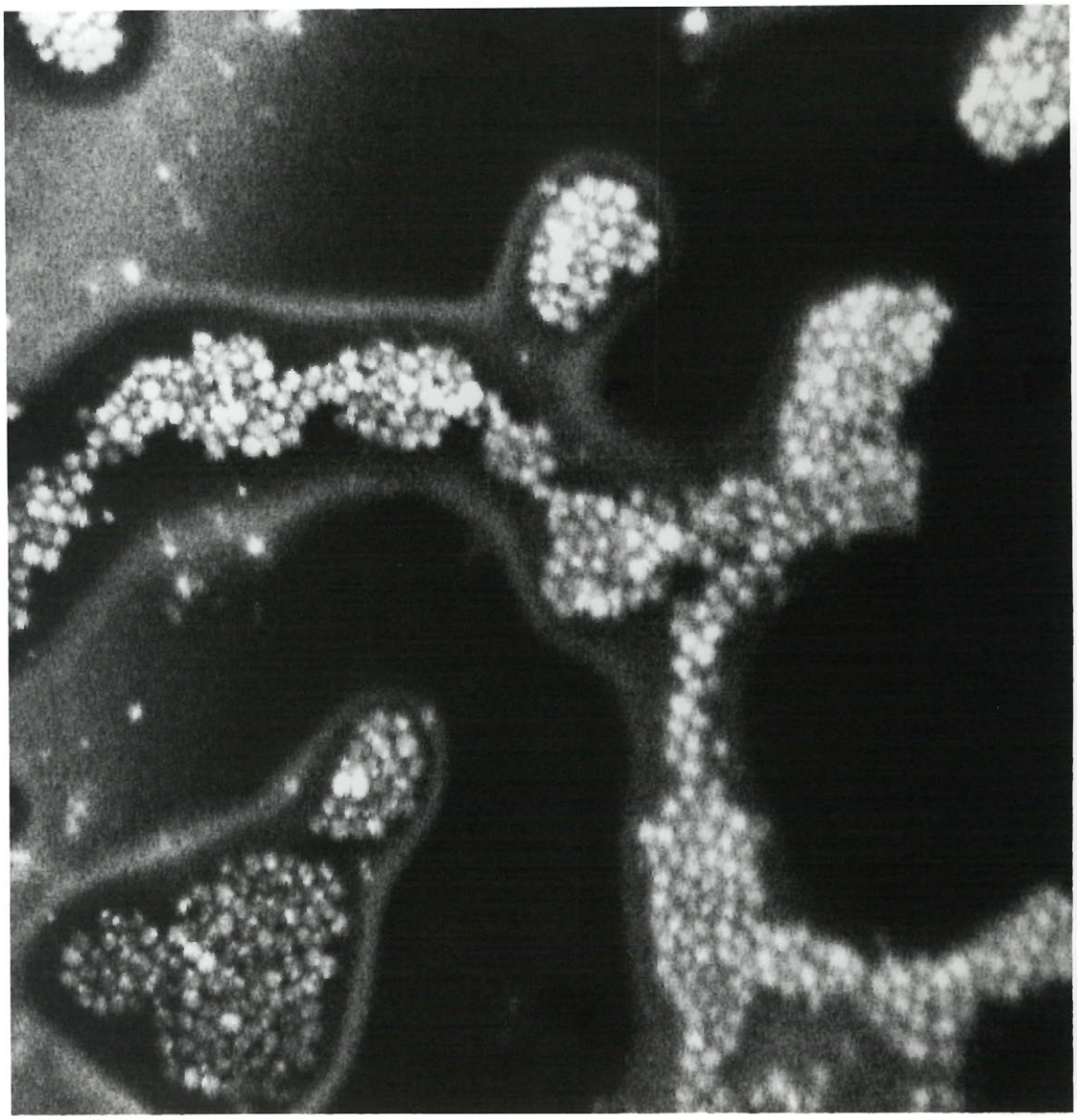


Figure 11b. Higher magnification of photomicrograph of Figure 11a (SCLM, magnification X 2925).

Clusters of normal appearing cocci are seen outlined by the clearly visible extracellular matrix.

Notice the sac-like appearence of the continuous biofilm matrix closely molded around the bacterial clusters. The biofilm matrix lies in close proximity to the bacterial clusters, supporting the possibility of the involvement of the matrix acting as a shield to the bacteria against antibiotic action and host defense mechanisms.

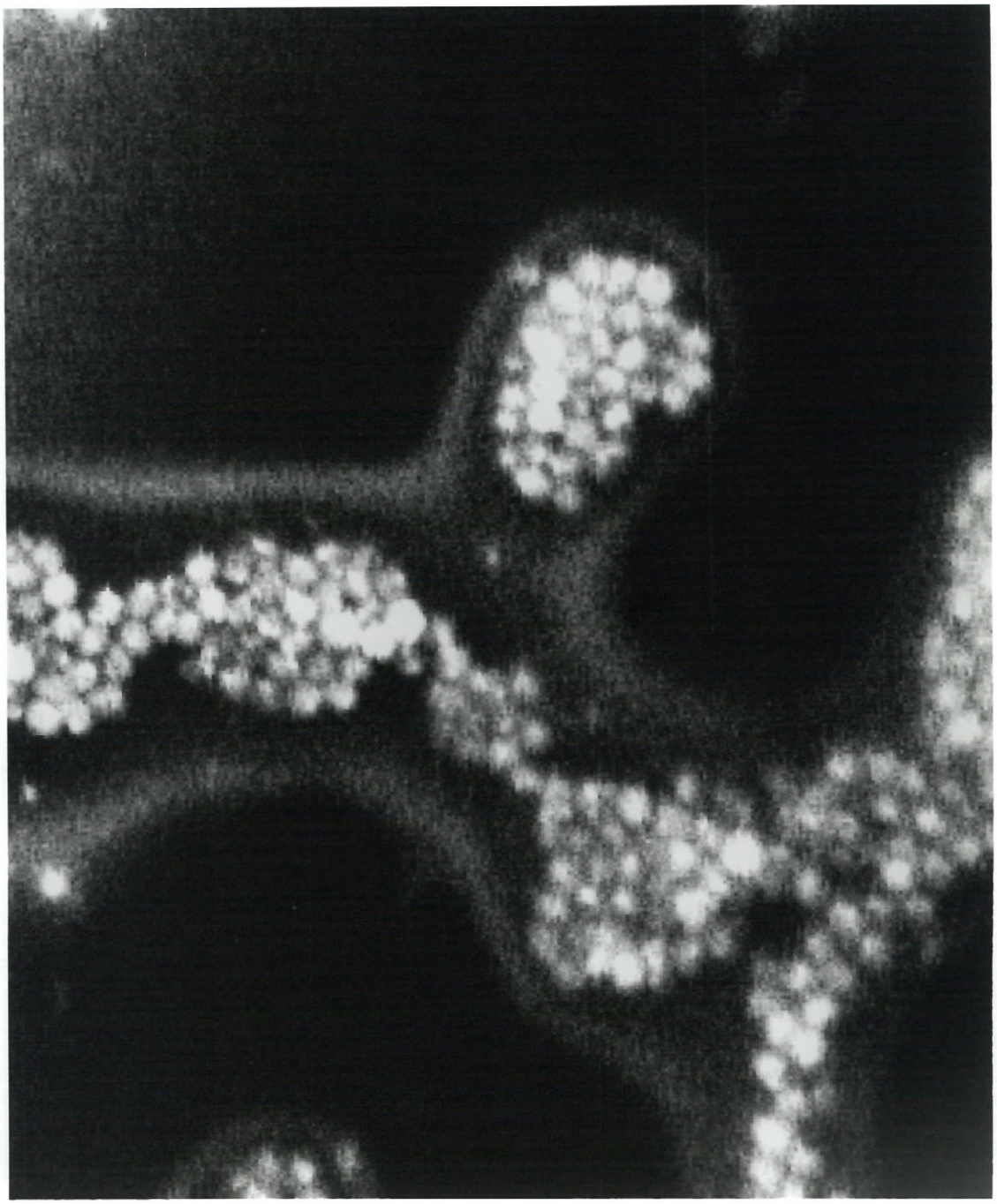


Figure 11c. Photomicrograph of another area of the control S. epidermidis biofilm (MGH #2503) presented in Figure 11a (SCLM, magnification X 1050).

Numerous cocci are seen amidst large areas of extracellular matrix.

Notice the contrasting morphological appearence with Figure 11a with less clustering and denser broad areas of matrix. In places (lower part) the matrix is so dense that it forms a solid white band with sharply demarcated border.

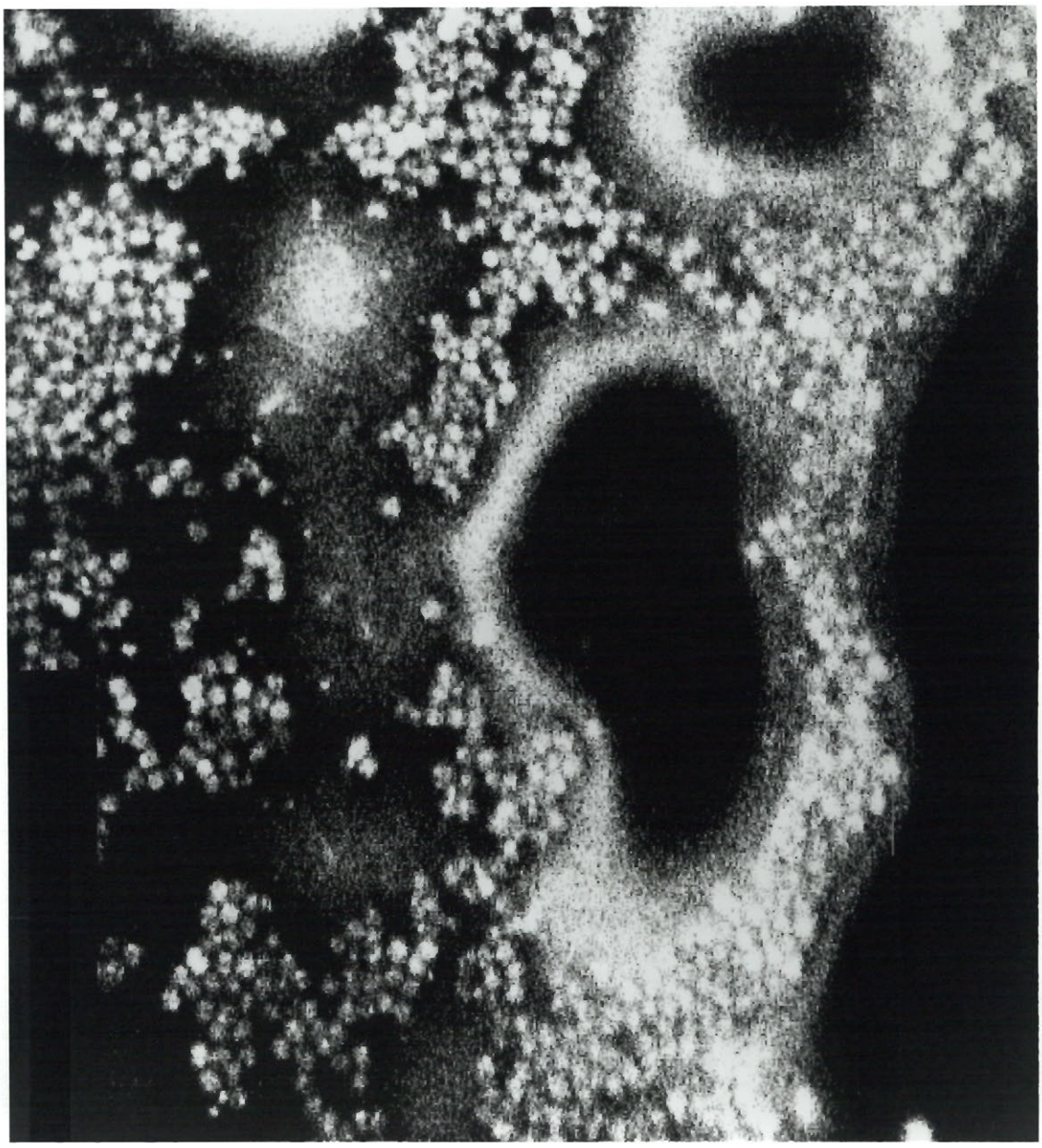


Figure 12a. Photomicrograph of an experimental Staphylococcus epidermidis biofilm (MGH #2503) on a glass surface following antibiotic exposure. Images obtained using a scanning confocal laser microscope (SCLM, magnification X 1050).

The biofilm was exposed for 24 hours to gentamicin (10 μg/ml), an aminoglycoside antibiotic. The biofilm was immediately prepared for the SCLM examination by short-term exposure to FITC, a fluorescent dye. The bacteria fluoresce brightly while the biofilm matrix is perceived as a separate low fluorescent zone clearly distinct from the black background.

Generalized disorganization of the biofilm is seen with marked alteration of the bacterial population and widespread disappearence of the extracellular matrix.

Notice the heterogeneity in the biofilm morphology with a more advanced state of destruction seen in the lower part. There, the bacteria aggregate in clumps of various sizes and the matrix is absent. In the upper part, evidence of abnormal bacterial clusters in an apparently preserved matrix can still be recognized. Notice also the widespread abnormal bacterial morphology with pleiomorphism in size, shape and degree of fluorescence.

It is noteworthy that the gentamicin-exposed biofilm while showing marked structural changes after 24 hours exposure, still retains considerable metabolic activity as assessed by the TTC method (Figure 10).

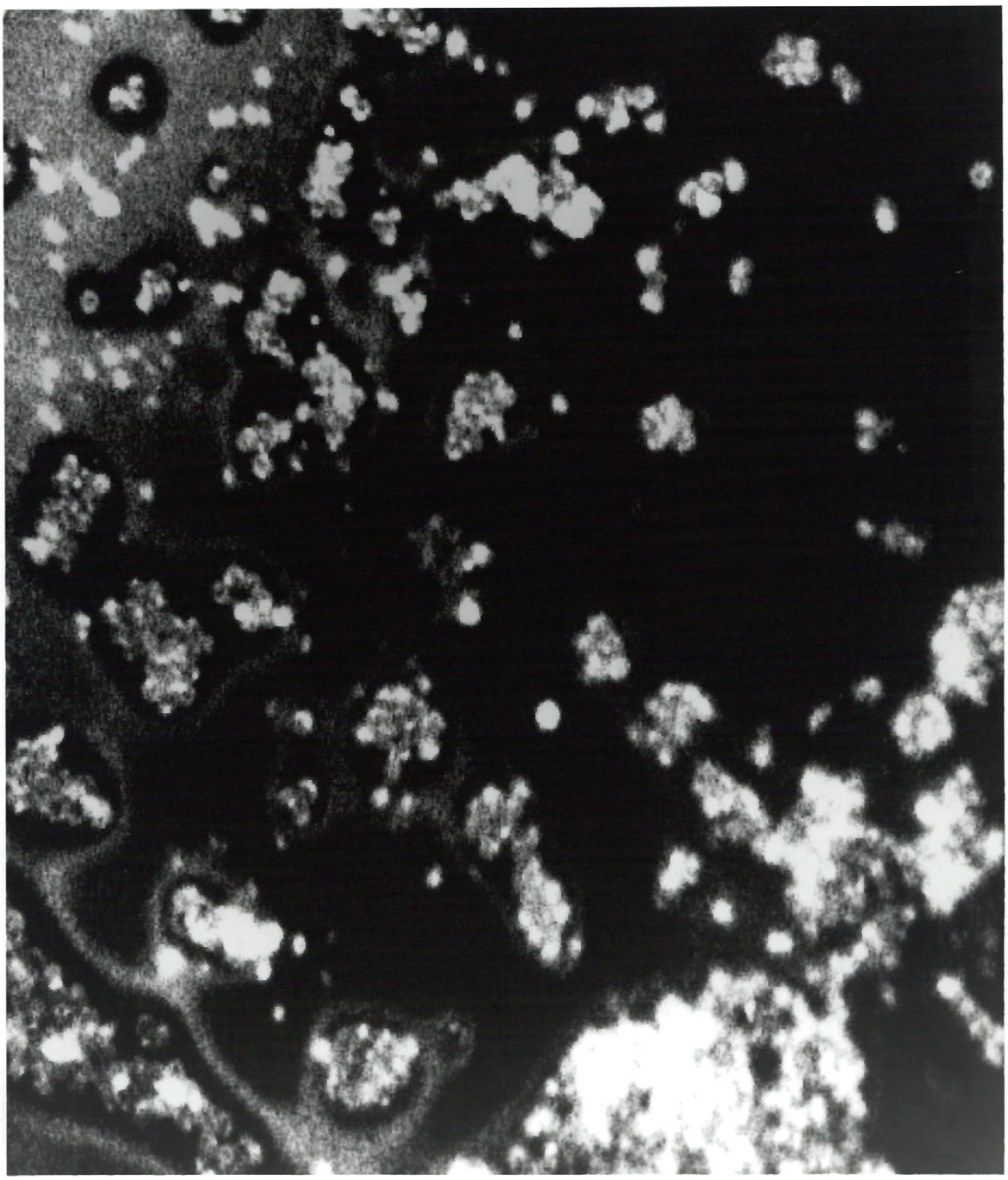
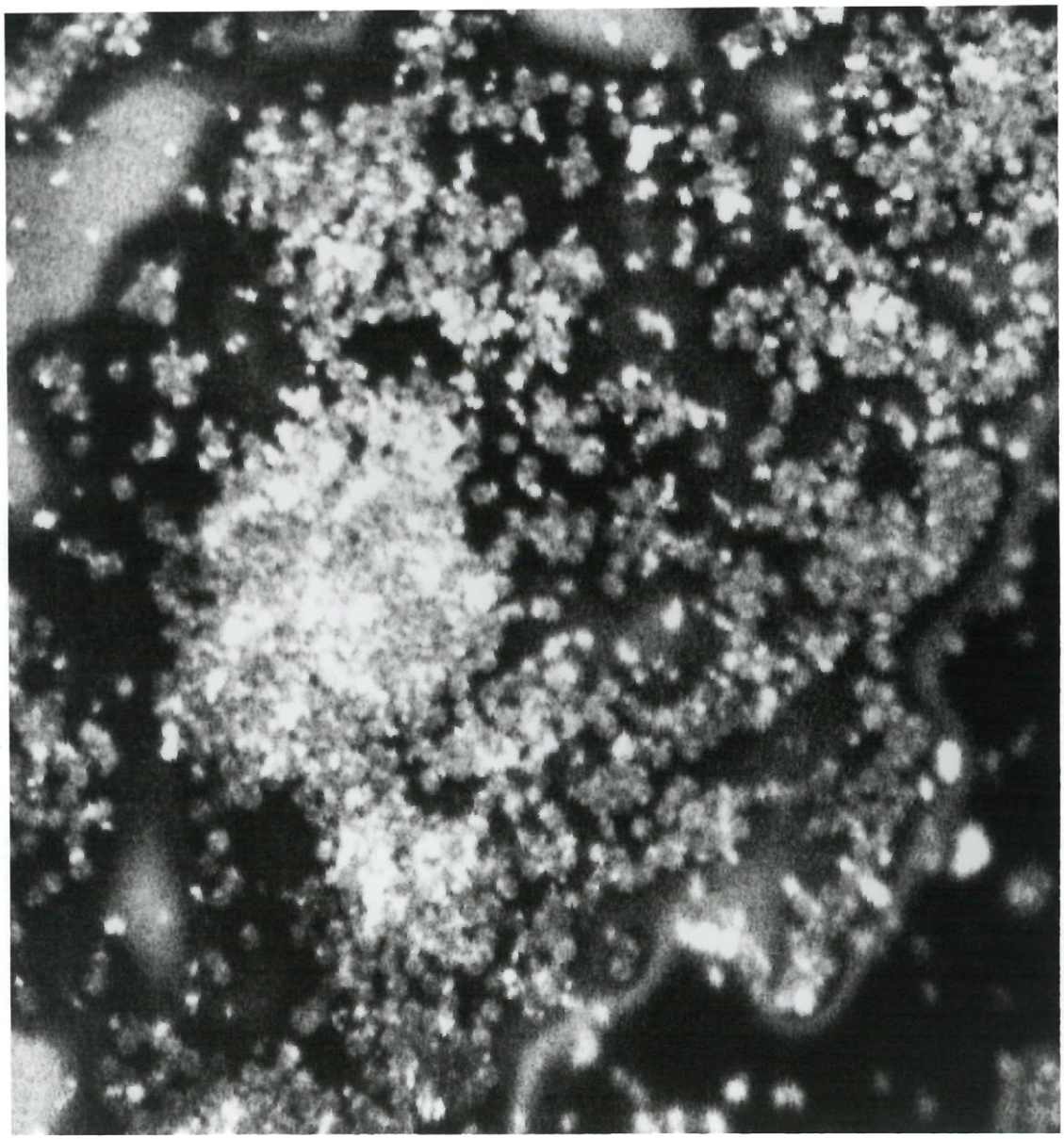


Figure 12b. Photomicrograph of another area of the antibiotic-treated S. epidermidis biofilm (MGH #2503) presented in Figure 12a (SCLM, magnification X 1050).

Notice the advanced state of destruction of the biofilm still containing large bacterial numbers with loss of clustering replaced with large clumps of abnormal looking bacteria and widespread disappearance of the matrix.



SUMMARY OF RESULTS

1. Experimental Staphylococcus epidermidis biofilms demonstrate solid resistance to high concentrations of commonly used antibiotics with the exception of rifampin.

2. Rifampin displays rapid extensive antimicrobial activity against experimental S. epidermidis biofilms; its action, however, is incomplete with the persistence of rifampin-resistant foci which readily repopulate the biofilms.

3. The resistance of experimental S. epidermidis biofilms to antibiotics other than rifampin is not absolute and is overcomed by increasing concentration and time of exposure.

4. Many antibiotics (particularly cell-wall active agents) complete the predominant effect of rifampin on experimental S. epidermidis biofilms while others (several aminoglycosides) inhibit rifampin action. These synergistic and antagonistic interactions with rifampin occur in double and triple combinations.

5. Peritoneal dialysis solutions complete the antimicrobial activity of rifampin against experimental S. epidermidis biofilms. This synergistic effect was comparatively faster for fresh PD solutions than for spent PD fluids obtained from CAPD patients.

6. The most common non-antibiotic therapeutic additives to PD solutions, heparin and insulin, do not affect the antimicrobial activity of antibiotics against experimental S. epidermidis biofilms. Urokinase in contrast accelerates the slower synergy of spent PD fluids to rifampin.

7. Fully hydrated experimental S. epidermidis biofilms studied by SCLM show distinctive structural organization with characteristic features of the bacteria and the surrounding matrix in the control intact state. Both these components are strikingly altered upon effective antibiotic action.

8. Experimental S. epidermidis biofilms prepared with a derivative of the standard strain resistant to rifampin in the fluid phase present broad resistance to all antibiotics, including rifampin.

DISCUSSION

The clinical application of biomaterials has expanded exponentially in recent years (164). There is an estimated annual use of more than 2 billion biomaterial devices in therapeutic medicine and the expectation of a further steep increase in their application. Infection is the main complication of implantation and often presents unusual resistance to standard anti-infective regimes. The immediate, short-term, and long-term infectious complications of medical implants require an accelerated understanding of the interactions of biomaterials and microbes with special emphasis on the antibiotic resistance of implant-associated infections.

Progressive renal failure left untreated leads to death. Replacement of renal function may be undertaken by renal transplantation (limited by the availability of matching donor kidneys and the health of the recipient) or by dialysis. Peritoneal dialysis is a preferred mode of dialysis for many, and with an ageing population is increasing in use exponentially (39).

The major limitation of CAPD is infection, which occurs primarily in association with the implanted peritoneal catheter and may spread to a diffuse peritonitis. Such infections, particularly if repeated, may lead necessarily to abandonment of this desirable mode of replacement therapy (42, 82, 115, 138).

Catheter-associated infections have a number of characteristics which sharply separate them from infection which arise in the absence of foreign bodies (Table 3). Clinically, infections associated with implant material tend to become persistent and chronic, indicating a protective mechanism shielding the bacteria from host defenses. Furthermore the infections are noted to be slow or fail to respond to antimicrobial therapy even when laboratory investigations indicate an expected prompt and successful response.

A clue to the process which is taking place is given by the nature of the infecting organisms which is significantly different in species distribution from those commonly causing infections in the absence of implant material. The shift is towards skin-derived species, such as S. epidermidis which, in the absence of immunosuppression or foreign material, is regarded as essentially non-pathogenic. It is clear that it is the presence of the implant surface which mediates the protection given to the invading bacteria. If the implant is removed, the infection resolves.

In the environment of natural surroundings, microbiologists have long recognized that bacteria can exist in one or other of two forms (27). In one form, the biofilm or sessile state, the bacteria have attached to surfaces and then generate a slime-like protective coating for the enclosed bacterial community by polymerization of available environmental nutrients by extra-cellular enzymes. In the second form, which is more familiar to applied medical microbiologists the bacteria exist in a free-living and susceptible planktonic phase, migrating rather like seeds to find a favorable environment before converting on a surface to the shielded, sessile biofilm phase.

It is only in the last decade that serious attention has been given to the concept that implant-associated infections may be understood as a protected biofilm form of bacterial organization developing on the implant surface (30).

The demonstration, by electron microscopy, that the biofilm phase of bacterial organization exists in Man on the surfaces of implanted devices implicated in infection, strengthens this view (94, 95, 113). So, too, is the discovery that S. epidermidis can be a notable slime-producer by extra-cellular enzyme action, forming a slime-like protective coating for the enclosed bacterial community by polymerization of available nutrients in the environment (114). This property is strongly associated with the ability to cause clinical disease.

The current attractive theory to explain the characteristics of implant-associated infections is that they are due to the biofilm phase of bacterial existence and that it is the biofilm matrix which, by selective permeability, permits the access of bacteria to nutrients but at the same time shields them from potentially harmful anionic environmental substances. These include the majority of antibiotics, excepting rifampin which is a zwitterion, and the elements of host defense mechanisms.

With this concept it is seen that an in vitro model of implant infection, comprising the implant surface - bacteria - matrix complex will, if standardized, permit the study of those variables suspected of modifying the crucial nature of matrix permeability to permit optimization of entry of antimicrobials and host defenses.

The in vitro model used in this investigation consisted of a standardized S. epidermidis biofilm formed on glass. Two bacterial strains were used. A constant, slime-producing strain (MGH #2503) was originally isolated from a patient with an intravascular device infection and has been characterized in detail without change of properties over several years of study (106). It is fully sensitive to all relevant antibiotics, including rifampin, by routine fluid phase susceptibility testing. The second strain (#2503RR) is derived from MGH #2503 and is a stable mutant or daughter strain identical in character to the original with the exception of solid rifampin resistance.

The detection of microbial metabolic activity was demonstrated by the ability of the S. epidermidis bacterial cells enclosed within the biofilm to reduce the colorless 2,3,5-triphenyltetrazolium chloride (TTC) to a deep red insoluble formazan (4, 6, 54). The measure of the relative impacts of changing hostile environments on the metabolic activity of the biofilm preparations was the time taken (measured in days) for metabolic activity to be reduced to a permanent

standstill i.e. bacterial cell death. This quantal end-point of sterilization is of clinical relevance, since it is the intention of the management of implant-associated infections to ensure that there are no bacterial survivors on the implant surface that might permit infection to re-establish when once antimicrobial therapy has been discontinued.

The apparent gain in information and speed by defining the proportional rate of bacterial cell death using a quantitative measure which does not continue to a statistically definitive outcome (i.e. complete kill) may not be necessarily advantageous. There is a temptation to extrapolate a perceived tendency and thereby incorrectly predict a successful outcome to save time waiting for the conclusive demonstration of bacterial biofilm death. The described assay, however, requires that the quantal end-point be seen to be a complete cessation of bacterial metabolic activity in the test biofilm. This end-point may take longer to determine but is secure and foolproof.

The bacterial biofilm assay utilized in this study demonstrated that standardized S. epidermidis biofilms display the major characteristic of implant-associated infections which is general resistance to antibiotics at clinically achievable levels. The assay forms an in vitro analogue of this class of infection and is amenable to modulation, thereby providing a valuable experimental tool to study this phenomenon. The initial findings obtained in this study confirmed some of the observations previously made in experimental investigations of CAPD catheter-related sepsis (33, 113, 126).

The experimental S. epidermidis biofilms displayed a high metabolic rate indicating free entry of essential nutrients from the environment. A broad resistance to the penetration of antibiotics, with the single exception of rifampin, was found indicating selective blocking of anionic molecules. This finding

parallels clinical and laboratory observations on the problem of management of implant-associated infections.

There are a number of important variables which must be evaluable in an effective assay of antimicrobial activity. These include the nature of the implant material (composition, charge and surface properties) and of the infecting organism (density, adherence and susceptibility to antibiotics) since they affect the initiation, degree and outcome of the infectious process. Clinical factors which modify therapeutic outcomes include the choice of an appropriate antibiotic or combination of antibiotics, the dosage levels and the duration of therapy. Furthermore, the interactions of the host environment with the bacterial species, implant surfaces, antimicrobial activity, therapeutic agents other than antimicrobials and non-physiological challenges with fluids such as PD solutions, must all be capable of independent examination.

The technique of exposing standardized bacterial biofilms formed on a standardized template surface to a milieu of varying complexity permits the attainment of these aims. The chosen method has the simplicity and directness of using an end-point which can be read by eye, requiring only routinely available equipment and supplies of an elementary laboratory facility, measuring the time required to reach a state of specific clinical relevance - in this case bacterial biofilm death (sterilization of the implant surface).

Routine antimicrobial sensitivity tests utilize bacteria in low concentration in the susceptible actively-growing fluid phase. Bacteria are deemed to be sensitive to an antibiotic if bacterial multiplication is inhibited by a concentration of antibiotic achievable in blood or tissue fluid. Infections managed with appropriate antibiotics selected on the basis of such susceptibility test results are regarded as likely to lead to a satisfactory clinical outcome.

However, the management of implant-associated infections based on routine susceptibility test data is commonly unsuccessful. It is being increasingly accepted that the presence of the implant material is influencing the host-microbe-antibiotic interaction and in some way interfering with the expected activity of the antimicrobial therapy. In addition it is seen that clinical success requires more than just inhibition of bacterial growth but rather a complete elimination of bacteria to avoid relapse when antibiotic presence is removed.

Tests of antibiotic efficacy utilizing standardized S. epidermidis biofilms indicate that many antibiotics have much slower rates of action than is indicated by standard fluid phase testing, that significant differences exist in rates of action amongst antibiotics and that unsuspected significant antagonisms and synergistic interactions can be exposed (128, 135).

The concept that the clinical resistance of implant-associated infections to host defenses and antibiotic therapy is due to the properties of the extracellular polymer substances formed by bacteria in contact with foreign-material surfaces is attractive. Study of the biofilm matrix in vitro should therefore confirm differences in rates of actions of different antibiotics as is noted in clinical practice and thereby allow the rational in vitro optimization of combination antibiotic therapy by direct experiment (48, 132, 134)

Rifampin has a singular action compared to the 30 other antibiotics studied in this laboratory (106). In general, the period of exposure for antibiotics required to sterilize susceptible S. epidermidis biofilms is measured in days. For rifampin (10 μg/ml) the numbers of residual viable bacteria approach zero within 8 hours (108). A biofilm tested at that time will appear to be dead: it requires further culture to reveal the presence of foci of rifampin-resistant survivors. These have arisen by spontaneous mutation which occurs in any

substantial population of staphylococci at a frequency of approximately one in 10^7 divisions. This phenomenon of rapid, nearly complete action suggests the utility of using rifampin activity as a sensitive tool to assess the interactions of other antibiotics and CAPD variables on biofilm permeability. An acceleration of action or the completion of rifampin bactericidal outcomes would indicate synergy whereas the opposite would indicate antagonism.

The question arises as to the properties that rifampin possesses that enables its striking rapidity of action. The majority of antibiotics have molecules that are electronegatively charged and it is known that the overall surface charge of the biofilm matrix is also electronegative. It is reasonable to assume that it is electrostatic repulsion that accounts for the ability of the biofilm to impede the entry of most antibiotics.

Rifampin is a zwitterion - a molecule in which the bonds between the constituent atoms are in a state of flux so that an electron alternates from one side of the molecule to the other (136). The aspect of the molecule presenting to a surface is therefore 50% of the time electropositive and thereby gains admittance through the biofilm matrix electronegative field.

The physical structure of the biofilm which gives rise to its selective properties has been the subject of speculation. It has been advocated that the polymer molecules of the matrix are oriented in a stack in order to give the surface its intense electronegative charge which will repel all noxious molecules of similar charge. An alternative view holds that the matrix is permeated by micro-channels. It is the dimension of these channels plus the surface charge effects (acting as a cationic trap) that exclude the larger anions yet permit the free ingress of small molecule nutrients. Yet another speculation proposes that micro-channels exist, penetrating the matrix from surface to bacteria, analogous to the porin channel construct of bacterial cell membranes,

where non-selective channels (for nutrients) exist side-by-side with selectively blocked channels which can exclude potentially noxious elements of the environment.

These views would imply that the matrix possesses an absolute barrier - that certain molecules would be accepted, while others would be excluded except under the most abnormal of environmental challenges. Indeed, the testing of biofilms with antibiotics over short periods e.g. 12 to 18 hours does show that, in order to effect sterilization, concentrations that greatly exceed those permitted in Man would be required.

This study demonstrates that there is a steady, progressive relationship between concentration of agent and the duration of exposure required to bring about sterilization. This observation indicates that the selective property of the biofilm is not absolute but rather an impedance which is overcome by constancy and concentration gradient. It suggests that the structure of the biofilm is different from that previously proposed.

Naturally occurring polymer molecules can adopt one of three basic structures - the spiral, the straight rod and the kinked rod (19). Electron microscopy of dehydrated, fixed biofilm matrices reveals it to be composed of rod-like fibrils (18). The structure which best fits the finding that the matrix possesses a non-absolute selective permeability, in which the rate of penetration of antibiotics is governed by a concentration-time relationship, and in which the thickness of the matrix alters the rate of penetration - is a simple mat of randomly placed polymer rod-fibrils with an overall electronegative charge.

A domestic analogue would be the air-filter mesh in the air intake of a hot-air furnace. A simple fibre mesh which permits air to pass through but

retains particles by electrostatic charge. The thicker the mat, the greater the efficacy in particle retention.

At the chosen antibiotic test concentration of 10 μg/ml the data derived from these studies confirm that significant differences do exist between different antibiotics (Table 9a). All selected antibiotics used singly will take at least 3 days, if at all, to sterilize the test S. epidermidis biofilm preparations. Rifampin has a superior rate of action but, as has been noted clinically, resistant bacteria are selected and neutralize the initial bacterial reduction.

Rifampin activity against S. epidermidis biofilms in vitro is incomplete because of the development and selection of rifampin-resistant cells which are present in any population of rifampin-sensitive staphylococci, arising by spontaneous mutation at a steady low frequency. The rifampin-resistant mutants persist and multiply despite the presence of rifampin at high concentrations. It is known however, that although incomplete in action, rifampin exerts its initial activity very rapidly bringing about a reduction of viable bacterial cells by 99.9% within the first 8 hours of exposure. Rifampin therefore is accepted as a basis of effective antibiotic combination therapy because of the speed and magnitude of its effect, but may not be used alone. The key problem is to determine what modulation is required to bring about a total and rapid kill.

The selective permeability of the rifampin-sensitive S. epidermidis biofilms having first been damaged by rifampin, permits otherwise excluded representatives of the penicillin-first generation cephalosporin group of antibiotics and vancomycin to gain access to the biofilm bacteria and eliminate the scanty rifampin-resistant survivors with overall enhanced rates of action (Table 9b). This finding accords with the current clinical views of the value of adding rifampin to antistaphylococcal antibiotic regimes for implant-associated infections (42, 116).

A number of antibiotics have little or no detectable change in their rates of action when combined with rifampin. In sharp distinction, gentamicin, unlike other members of the aminoglycoside family, clearly antagonizes the action of rifampin. This in vitro observation gives rise to concern since gentamicin is an antibiotic commonly used in antibiotic regimes which include rifampin to treat persistent staphylococcal infections. These in vitro findings mandate further clarification to determine if the antagonism is also a disadvantageous in vivo manifestation.

The aminoglycoside group of antibiotics are so named since they are characteristically composed of an aminocyclitol glycosidically linked to an aminosugar. The group is divided into two main families according to the structure of the contained aminocyclitol, whether it is a streptidine or a deoxystreptamine (127). Streptomycin is unique since it is the sole therapeutic representative of the streptidine family and is structurally distinct from the deoxystreptamine family which contains the five other aminoglycosides used for this comparative study. The various differences in structure determine the activity of the antibiotics. This difference in structure and corresponding expected differences in biological activity is strikingly confirmed by the data of Tables 9b and 10. A notable finding is the divergence of action of streptomycin (a streptidine) from the other aminoglycosides (deoxystreptamines). This division of aminoglycoside action had not previously been suspected, and may prove of significant clinical value.

The present study substantially extends earlier studies, which demonstrated the synergy of fresh PD solutions with rifampin against S. epidermidis biofilms, by providing information about solutions other than dextrose-based lactate-buffered solutions and about rifampin-resistant S. epidermidis biofilms. This study showed constant synergy of all fresh PD

solutions with rifampin when used either alone or in combination (with gentamicin). It is still not known, however, how the PD solutions cause this synergy or why this synergy is selective for rifampin. A possible explanation resides in the "unphysiological" characteristics of the solutions which might kill the foci of rifampin-resistant survivors after the rapid predominant action of rifampin on the biofilms. An argument for a pH effect of the solutions on biofilm bacteria is the superior synergy demonstrated by the dextrose-bicarbonate solution of higher pH well-known to be detrimental to bacteria in the fluid phase.

A possible concern with dextrose-based PD solutions is that the high dextrose content might promote exopolysaccharide production by the biofilm bacteria contributing to the antibiotic resistance. No significant difference could be found in antibiotic action in the presence of any of the lactacte-buffered PD solutions whether the osmotic agent was dextrose or not, indicating the absence of significant difference in biofilm formation in these different environments.

The consistent finding of the weaker synergy of spent fluid with rifampin compared to fresh PD solution is of considerable clinical importance since the fluid in the peritoneal cavity of CAPD patients always shares characteristics with spent fluid. When fresh PD solution enters the peritoneal cavity, complex changes in its composition progressively occurs (104). It is immediately mixed with the fluid in the peritoneal cavity, which is remaining from the end of the previous cycle and thereby straightaway loses the characteristics of fresh PD solution. As the dwelling period extends the composition of the PD fluid changes continuously towards the final characteristics of spent fluid. The composition is altered as a result of the concurrent action of the two main forces of ultrafiltration (resulting in a rapid gain of water from the effect of the osmotic agent), and of diffusion (resulting in a slower gain of small molecular solutes

specifically those retained in renal failure). The composition is further altered by progressive dissipation of the osmotic agent by peritoneal absorption and gain of large molecules such as proteins. The finding that fresh PD solution has greater synergistic interaction with relevant antibiotics suggests the desirability in the management of CAPD-associated peritonitis of more frequent exchanges of PD solutions or of constant cycling.

The weaker synergy of spent fluid to rifampin compared with fresh PD solution warrants explanation. It is difficult to ascribe this difference to a single factor since the composition of the two fluids differs in so many respects (Table 14). A possible approach to identify the element or combination of elements responsible for the slower synergy of the spent fluid would be to test an in vitro "reconstituted" spent fluid starting step by step from a fresh PD solution.

Although the roles of PD solutions, both fresh PD solution as well as spent fluid, have been investigated extensively, less attention has been paid to the significance of non-antibiotic therapeutic additives to PD solutions on host-bacteria-implant surface interactions. Heparin is no longer considered to be an innocent by stander in the initiation and propagation of coagulation associated with foreign surfaces (66, 148). Insulin possesses broad regulatory properties which can come into play in cellular immune responses elicited by the presence of a foreign body (77, 105). The fibrinolytic agent urokinase also can participate in vivo given the recognized ability of artificial surfaces to initiate the coagulation process and the deposition of fibrin (10, 31).

In this study heparin and insulin were found not to affect rifampin activity whereas urokinase unexpectedly completed rifampin action in the presence of spent PD fluid. The mechanism responsible for this effect of urokinase remains to be determined. Spent fluid contains large molecules including fibrin which is

readily formed through the activation of clotting mechanisms by biomaterials. It is possible that fibrin, through as yet unknown mechanisms, prevents the entry of antibiotics in bacterial biofilms. Urokinase by its fibrinolytic action will destroy fibrin, thus promoting antimicrobial effect. Clinical evidence for a beneficial effect of adding urokinase to the standard antibiotic treatment of CAPD peritonitis would support this hypothesis (73, 117, 118)

A further prime objective of this study was to correlate the metabolic activity of the S. epidermidis biofilms under defined experimental conditions with the morphological appearance of both matrix and enclosed bacteria. These morphological studies were performed by scanning confocal laser microscopy because biofilms are highly hydrated and consequently of the advantages of avoidance of dehydration and artifacts produced by imposed bacterial damage and death. Other current forms of microscopy necessarily require dehydration and will induce structural changes (16, 19, 157).

These confocal microscopy structural studies were undertaken on biofilms which had been exposed to planned environmental extremes: the environment of peptone water sustaining full metabolic activity of the biofilm as a control and the environment of the antibiotic gentamicin (10 μg/ml) which leads to cessation of metabolic activity by 3 days (Table 8).

In the control experimental situation (peptone water environment) SCLM of the S. epidermidis biofilms revealed the outstanding images of cocci growing in clusters enclosed in an extracellular matrix. The well identified cocci (brightly fluorescent spherical forms) were seen within a paler fluorescent dense acellular matrix, confirming the predicted close ordered relationship between the two biofilm components.

Following 24 hours of exposure to gentamicin, SCLM of the S. epidermidis biofilms revealed the striking images of severe disorganization

of the biofilms, with changes affecting both the bacteria and the surrounding matrix. Extensive disappearance of the matrix was evident with marked morphological alterations of the remaining bacteria. Additional SCLM examination is required to delineate the sequence of development of these structural changes.

In summary, experimental S. epidermidis biofilms were found to be relatively resistant to antibiotic action, with the single exception of rifampin.

This antibiotic resistance was not absolute and could be overcome by increasing antibiotic concentration and time of exposure.

Unexpected interactions were observed between members of the aminoglycoside class of antibiotics and rifampin.

Streptomycin showed strong synergy with rifampin whereas several other aminoglycosides, gentamicin in particular, antagonized rifampin action. These divergent effects are explained on the basis of structural differences.

Fresh PD solutions were synergistic with antibiotic action in general. Spent PD fluid had a lesser synergistic action. Amongst the various formulation of PD fluids, dextrose with bicarbonate showed the optimal synergy. Cloxacillin, cefazolin and streptomycin were optimally synergistic in combination with rifampin.

The demonstration of significant modulation of microbial responses to alterations in the peritoneal environment confirms the initial hypothesis.

During the course of the project, several difficulties were encountered. For example, despite strict adherence to the described technique, a minor interassay variation was noted in the antimicrobial activity of certain antibiotics. This variation which was ascribed to different biofilm density did not prevent significant observations to be made on antibiotic action against S. epidermidis biofilms. Moreover the rifampin-resistant S. epidermidis strain (#2503RR) did

not readily form a biofilm under the conditions of the assay used for the rifampin-sensitive S. epidermidis strain (MGH #2503), but this could be resolved by increasing the glucose concentration of the growth medium and prolonging the incubation time. The variability in pH of the dextrose-bicarbonate fresh PD solution posed an additional difficulty; however, the results of several assessments conducted with this solution at different measured pH were all in concordance. Another difficulty was the inability to study spent fluids of CAPD patients on presentation with peritonitis because of the unpredictable timing of this acute clinical event. A number of technical difficulties were encountered initially with SCLM examination because of the lack of information available. The images obtained by this technique of this class of S. epidermidis biofilm preparations are the first to be published.

Despite the difficulties encountered, the aims of the research project were effectively met: the exploration of antibiotic activity against S. epidermidis biofilms as an analogue of CAPD-associated infection and the exploration of the outcomes of modulation of antibiotic activity by CAPD variables.

An important consideration in the management of chronic infection is awareness of the impact of increasing antibiotic dose on the rate of sterilization. Data is required in order to justify increasing medication to maximum levels or conversely declining to risk toxic complications if there is no benefit to be obtained.

All in vitro tests of antibiotic susceptibility which attempt to predict clinical outcomes in the management of infection are limited in attempting to imitate the disease process. Tests of antibiotic action which measure the relative efficacy of bacterial biofilm penetration are necessarily also limited. The relevance of these new predictive techniques to the clinical situation of implant-associated infections requires detailed study in a controlled experimental environment. If

there is a closer correlation between in vitro predictions and in vivo outcomes then such new forms of testing will represent a significant technical advance.

The results obtained in this investigation require further studies in several different directions. The following experiments are a natural extension of the present study:

(a) To conduct a similar assessment of antibiotic action against experimental biofilms of S. aureus, the second most frequent pathogen associated with implantation after S. epidermidis.

(b) To determine the kinetics of the morphological changes (using SCLM) in S. epidermidis biofilms after exposure to various milieus relevant to the CAPD situation. The disintegration of the protective biofilm matrix observed microscopically will be a key marker.

(c) To utilize SCLM and fully document the sequential changes following exposure of biofilms to rifampin, from the early predominant kill to the late repopulation with rifampin-resistant survivors.

(d) To assess the effect of spent fluid obtained from CAPD patients during episodes of peritonitis on the antimicrobial activity of antibiotics against S. epidermidis biofilms to determine the predictive constituents responsible for synergistic outcomes.

(e) To compare the effect of spent fluids obtained from CAPD patients with high and low rates of peritonitis on the antimicrobial activity of antibiotics against S. epidermidis biofilms, to determine if constituents of PD fluids from such patients can be related to the susceptibility to infection.

(f) To explore further the synergistic effect of urokinase in spent fluid on the antimicrobial activity of antibiotics other than rifampin. Urokinase is an in vivo agent which has the potential for a beneficial impact on the thrombogenic activation, provoked by any foreign materials, regarded as the

prime precipitator of a chain of events leading to the onset of implant-associated sepsis.

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LIST OF PUBLICATIONS

A. Papers

1. Gagnon RF, Richards GK, Kostiner GB and **Morcos RJ**.
   Impact of dialysis fluid on the susceptibility of Staphylococcus epidermidis biofilms to rifampin.
   In: Advances in Peritoneal Dialysis-1993, edited by R Khanna, KD Nolph, BF Prowant, ZJ Twardowski and DG Oreopoulos.University of Toronto Press, Toronto, 1993, pp. 183-86.

2. Richards GK, **Morcos RJ** and Gagnon RF.
   The differential activity of aminoglycoside antibiotics with rifampin explored in a kinetic in vitro model of implant-associated infection (Staphylococcus epidermidis).
   In: Advances in Peritoneal Dialysis-1994, edited by R Khanna, KD Nolph, BF Prowant, ZJ Twardowski and DG Oreopoulos, Toronto: Peritoneal Dialysis Inc, Toronto, 1994, pp. 189-92.

3. Richards GK, Gagnon RF and **Morcos RJ**.
   An assay to measure antibiotic efficacy against Staphylococcus epidermidis biofilms on implant surfaces.
   ASAIO transactions. In press.

4. **Morcos RJ**, Gagnon RF and Richards GK.
   Impact of peritoneal dialysis solutions on the antimicrobial activity of antibiotics against experimental rifampin-sensitive and rifampin-resistant Staphylococcus epidermidis biofilms.
   In preparation.

B. Abstracts

- 13th Annual Conference on Peritoneal Dialysis, San Diego, California ,USA, March 7-9, 1993.

1. Gagnon RF, **Morcos RJ**, Richards GK and Kostiner GB.
   Staphylococcus epidermidis biofilms: The relationship of antibiotic concentration and rate of kill.

2. Richards GK, Kostiner GB, Gagnon RF and **Morcos RJ**.
   Impact of dialysis fluids on the rifampin resistance of Staphylococcus epidermidis biofilms.

• 39th Annual Meeting of the American Society for Artificial Internal Organs, New Orleans, Louisiana, USA, April 29, 30 and May 1, 1993.

3. **Morcos RJ**, Gagnon RF and Richards GK.
Comparative effect of glycerol and dextrose containing peritoneal dialysis solutions on antibiotic activity against Staphylococcus epidermidis biofilms.

• 62nd Annual Meeting of the Royal College of Physicians and Surgeons of Canada, Vancouver, B.C., Canada, September 10-13, 1993.

4. Richards GK, Gagnon RF, Kostiner GB and **Morcos RJ**.
The differential effect of fresh and spent dialysis fluid on rifampin activity against S. epidermidis in the biofilm phase.

5. Gagnon RF, Richards GK and **Morcos RJ**.
Comparative effect of peritoneal dialysis solutions on antibiotic activity against S. epidermidis biofilms.

• 14th Annual Conference on Peritoneal Dialysis, Orlando, Florida, USA, January 24-26, 1994.

6. Richards GK, **Morcos RJ** and Gagnon RF.
The effect of varying concentrations on antibiotic activity against Staphylococcus epidermidis biofilms.

7. **Morcos RJ**, Gagnon RF and Richards GK.
Relative effect of peritoneal dialysis solutions on antibiotic activity against Staphylococcus epidermidis biofilms.

• 40th Anniversary Meeting of the American Society for Artificial Internal Organs, San Francisco, California, USA, April 14-16, 1994.

8. **Morcos RJ**, Gagnon RF, Leclerc J and Richards GK.
Effect of low-molecular-weight heparin on rifampin activity against Staphylococcus epidermidis biofilms.

9. Richards GK, Gagnon RF, Prentis J, Kostiner GB and **Morcos RJ**.
An assay for the study of antibiotic kinetics against S. epidermidis biofilms.

• 63rd Annual Meeting of the Royal College of Physicians and Surgeons of Canada, Toronto, Ont, Canada, September 14-19, 1994.

10. **Morcos RJ**, Richards GK and Gagnon RF.
The differential activity of aminoglycoside antibiotics in combination with rifampin against S. epidermidis biofilms.

11. Richards GK, Gagnon RF, Kostiner GB and **Morcos RJ**.
Concentration-kinetics of antibiotics in synergistic combinations with rifampin against S. epidermidis biofilms.

• 27th Annual Meeting of the American Society of Nephrology, Orlando, Florida, USA, October 26-29, 1994.

12. Richards GK, **Morcos RJ** and Gagnon RF.
The divergent activity of aminoglycoside antibiotics in combination with rifampin against S. epidermidis biofilms.

13. **Morcos RJ**, Richards GK and Gagnon RF.
Comparative antibiotic activity against biofilms of S. epidermidis biofilms strains sensitive and resistant to rifampin.

14. Gagnon RF, Richards GK and **Morcos RJ**.
The differential effect of peritoneal dialysis solutions on antibiotic activity against rifampin-resistant and rifampin-sensitive S. epidermidis biofilms.

15. Kostiner GB, **Morcos RJ**, Gagnon RF and Richards GK.
Modulation by common drug additives to peritoneal dialysis solutions of rifampin activity against S. epidermidis biofilms.