TOPICAL IMMUNOTHERAPY FOR PSEUDOMONAS KERATITIS: USE OF ANTILIPOPOLYSACCHARIDE PLASMA

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ABSTRACT

Pseudomonas aeruginosa is an opportunistic pathogen capable of infecting the human cornea. Such infections are difficult to treat, and are often fulminative, in that the infected eye is lost, or severely scarred. The use of alternative therapeutic agents has been necessitated by the frequent failure of conventional antibiotic therapy.

Equine hyperimmune antilipopolysaccharide plasma (Anti-LPS) was obtained by the plasmapheresis of suitably immunized horses. The plasma contained 1,0-1,5g/ml of LPS-precipitible IgG antibodies. Topical administration of Anti-LPS as a lavage was shown to be effective against Pseudomonas keratitis in rabbits and guinea pigs. Subsequent use of topical corticosteroids was found to further reduce corneal pathology. The improvement noted in these experimental infections involved all three parameters measured, area of keratitis, depth of lesion, and degree of vascularization.

In vitro, Anti-LPS was shown to be rapidly bactericidal for Gram negative bacteria. The plasma can therefore be said to have a dual mechanism of action: antitoxic, and antibacterial.

Ocular administration of Anti-LPS, by both the topical and subconjunctival routes, was well tolerated by both rabbits and baboons.

In conclusion, Anti-LPS is a potentially useful immunotherapeutic agent with many applications in both veteriary and human medicine, particularly in the treatment of surface infections involving antibiotic-resistant Gram negative bacteria.

CHAPTER 1

1. INTRODUCTION

Pseudomonas keratitis is a serious bacterial infection of the cornea which is frequently very resistant to conventional antibiotic therapy. This disease has been described as "the most rapidly spreading and destructive bacterial disease the human cornea can be infected with as well as one of the most disastrous" (1). The aim of this project was to test a new therapeutic agent, equine hyperimmune antilipopolysaccharide plasma (Anti-LPS), against Pseudomonas aeruginosa, both in vitro and in vivo. To this end: 1) an experimental model of Pseudomonas keratitis had to be developed, 2) the safety of the product in this mode of utilization demonstrated, and 3) the effect of Anti-LPS on Pseudomonas characterised in vitro.

1.1 Epidemiology

Epidemiological features of Pseudomonas keratitis vary with the geographic situation of the host. In

El Salvador Pseudomonas aeruginosa was responsible for 34% of active corneal ulcers, mostly in healthy patients with ocular injuries (2). This was compared with the situation in at the University of California Hospital, San Francisco, where Pseudomonas caused 14% of such ulcers, usually in compromised hosts. Significantly, the final outcome of the infections was similar in both regions, despite the earlier and more specific therapy provided in the United States. In India Pseudomonas was found in 6,8% of corneal ulcerations, 5,1% of all post-operative infections, and was responsible for 5,1% of all positive cultures (3). Only 0,7% of normal control eyes harboured Pseudomonas, whereas 37% carried Staphylococcus epidermidis (responsible for 26,0% of corneal ulcers).Pseudomonas caused 11% of all corneal ulcers in a New York City hospital during the period 1950 to 1959, 6% from 1960 to 1969, and 8% from 1970 to 1979 (4). In comparison Staphylococci were responsible for 63% of all corneal ulcers during the period 1970 to 1979. They reviewed data reported for various areas of the United States, and noted an increased incidence

in warmer, more humid climates (37% in Florida, compared to 8% in New York). A review of microbial keratitis in Houston, Texas, showed that Pseudomonas caused 28% of cases seen from 1972 to 1981. Incidence figures therefore seem to vary considerably. An average incidence of about 15% can be expected. Although the incidence of Pseudomonas keratitis may be low in comparison to that caused by other bacteria, notably the Staphylococci, it is significant because of its poor prognosis. Although only 9 cases of bacterial endophthalmitis were reported to have occurred in a series of 16 000 cataract extractions, 2 were due to Pseudomonas, and both resulted in evisceration of the infected globe (5). It is the most common Gram negative bacillus infecting corneal ulcers (6).

With the widespread and increasing abuse of broad-spectrum antibiotic agents, an increase in the incidence of Pseudomonas related diseases has been noted. Only 100 cases of Pseudomonas keratitis had been reported in the world literature

up to 1965 (7). Recently, an outbreak of 6 cases was reported in one intensive care unit, all traced to careless intubation technique (8). The risks of antibiotic abuse have been well illustrated by studies of the changing patterns of drug resistance amongst pathogenic bacteria (9). In 1971, in New Delhi 80% of Pseudomonas isolates were sensitive to gentamicin (10). With increased use of this agent the situation changed dramatically; only 51,1% were sensitive in 1979, and 32,0% in 1980. Most of the resistant strains were isolated from corneal ulcers. A two-fold increase in the minimum inhibitory concentration (MIC) of carbenicillin and tobramycin for Pseudomonas was noted at MD Anderson Hospital, Houston , between 1977 and 1981 (6).

It appears that one of the consequences of more advanced medical procedures is an increased incidence of opportunistic infections, including Pseudomonas keratitis. As Froland has stated "the steadily increased use of cytotoxic and immuno-suppressive drugs, complex and invasive diagnostic and therapeutic procedures...can in several ways

pave the way for serious infections with opportunistic pathogens like Pseudomonas aeruginosa" (11).

1.2 Pathophysiology of Pseudomonas aeruginosa infections

1.2.1 Microbiology of the organisms

Pseudomonas aeruginosa is a Gram-negative aerobic saprophytic bacillus. It is motile, and has a varying number of flagella, in varied positions depending on the strain. It's natural habitat is soil and water. The genus as a whole is a plant pathogen (12), with only Pseudomonas aeruginosa being a significant human pathogen. One factor of extreme clinical importance is its lack of fastidiousness with respect to nutritive requirements. Almost purely aqueous media can be contaminated . An outbreak of Pseudomonas colonization in a cancer ward was traced to contamination of an ice machine (13). With specific reference to ocular infections, its ability to contaminate fluorescein solutions, saline, and various ocular medicaments has been responsible for many outbreaks, both

community-acquired and nosocomial (14). Prevention of such iatrogenic infections has improved, especially since the introduction of sterile fluorescein impregnated paper strips (15) and unit-dose eye drop presentation. It is also an extremely hardy organism, and is resistant to most commonly used disinfectants and antiseptics (16,17).

1.2.2 The opportunistic character of Pseudomonas

Opportunistic pathogens have been defined as "those microorganisms that are usually of limited virulence, but may readily invade, multiply, and produce disease in patients who have underlying conditions that decrease resistance to infection" (18). Pseudomonas aeruginosa is a clinically important member of this group. The host so predisposed to infection by an opportunistic pathogen can best be described as 'compromised'. Factors which may lead to a local or general impairment of the host's defence mechanisms are many and varied. Since extensive cooperation

occurs between the various aspects of the host defence mechanism, a combination of defects is often resposible. Each separate defence mechanism, and the factors affecting it which may predispose to infection with Pseudomonas aeruginosa, will be dealt with in turn. Although other disease states will be referred to, the main emphasis will be on ocular defences, and the factors predisposing to the development of Pseudomonas keratitis.

1.2.3 Host defence mechanisms

1.2.3.1 The epithelial barrier

By its compact, lipoidal nature, the intact corneal epithelium presents a physical barrier to bacterial invasion and infection. The outer layer of the epithelium is continually being sloughed off, and this, combined with the cleansing action of the tear film and blinking reflex, makes adherence of bacteria to the corneal surface very difficult. Pseudomonas aeruginosa has been found to adhere only to injured corneal cells and to exposed stroma (19). This seems to indicate that both microbial and host factors are involved in the adherence

process, i.e., there is a host receptor - bacterial factor interaction. Pilin from Pseudomonas fluorescens has been shown to bind to human corneal cells in a dose-dependent, saturable manner (20). This indicates either a limited number of binding sites, or steric hindrance to further binding due to the proximity of these sites, or the close apposition of different sites. Various reasons for the 'emergence' of these sites, following trauma to the cornea, have been proposed (19). The process seems to be mediated by bacterial pili. Any physical trauma to the cornea would thus predispose to infection. This can occur in a number of ways:-

1. eye injuries : perforating injuries of the eye occur mainly as a result of road accidents and child play (31 and 2% respectively, in a study at Birmingham and Midland Eye Hospital) (21). The remainder are contributed by occupational and domestic accidents, assault, outdoor activities, and sporting injuries. Socio-economic factors may however alter this distribution. A survey among Coloured patients at Groote Schuur Hospital, Cape Town, between 1977 and 1980, found that 59% of corneal lacerations were caused by assault, usually

with a knife or broken bottle (22). Eye injuries are of particular importance in the military sphere. In the 1973 Yom Kippur War in Israel, 5-8% of all combat injuries involved the eyes; 75% of all eye injuries were in personnel of the armoured corps (23,24). Peacetime military operations are also not without risk to the eyes - of 157 injuries, related to the use of 4 automatic weapons, seen at an American Army hospital between 1974 and 1980, 75 were eye injuries (25).

2. invasive surgery: any surgical procedure which disrupts the cutaneous integrity of the eye is potentially dangerous, because it provides a portal of entry for pathogenic bacteria. Infection during the procedure itself is highly unlikely. In an analysis of 16 000 consecutive cataract operations, only 2 cases of Pseudomonas endophthalmitis were noted. However, despite intensive therapy, both eyes were lost (5). Records of 36 000 cataract extractions over a 21 year period showed 31 infections, of all types. None of these was a delayed infection, so they could all be traced directly to the surgical procedure. Prophylactic regimens are therefore of vital importance, as

total sterilization of the operative field is virtually impossible (26-28). Postoperative infections have been reported after strabismus surgery, intraocular lens implantation, cataract surgery, closed vitrectomy, and penetrating keratoplasty (29-33). In these cases, the portal of entry was probably provided by the suture tracts or by wound dehiscence. Pseudomonas endophthalmitis has also occurred as a result of scleral necrosis caused by beta-irradiation following pterygium removal (34). Keratoplasty also carries the risk of contamination of the donor material, either from the donor or during storage in the eye bank (35-37). Pseudomonas cross-infection also occurs in other types of intensive surgery, as seen by an outbreak of Pseudomonas pneumonia reported in a cardiac unit (38).

3. contact lens wear : contact lenses have been described as "ocular foreign bodies", and as such present a considerable risk to the wearer (39). They can provide both the inoculum and the corneal injury necessary to initiate an often fulminative infection (40). Contamination of the

lens can occur during storage or insertion, as a result of inadequate aseptic technique (41). If correctly followed, the prescribed techniques are adequate, but this is seldom the case, especially after prolonged use (42). The following problem areas have been identified (39):

a) failure to reach sterilising temperatures when using thermal sterilization methods
b) loose fitting storage containers
c) contaminated solutions
d) contamination from the skin during handling

The primary problem seems to be the use of non-sterile saline, and its re-use over prolonged periods (43). The injury to the corneal epithelium can occur during a traumatic insertion or removal of the lens, caused either by the lens edge or the finger- nail. A more insidious form of corneal insult can occur as a result of friction between the corneal surface and protein deposits on the concave surface of the contact lens (44). Although Pseudomonas appears to be the dominant causative agent in these cases (41), other bacteria such as

the Serratia species can also cause similar sight-threatening infections (45). Varied incidence rates have been associated with different types of contact lenses (46-49). However, definite trends as regards possible increased risk associated with particular types of lenses have still to emerge.

4. use of ocular cosmetics : as with contact lens wear, the use of ocular cosmetics is a definite risk factor in the aetiology of corneal infections, including Pseudomonas keratitis. Many ocular cosmetics have been shown to support bacterial growth. These include mascara, powder and cream eyeshadow, and eyeliners (50). The major offender is mascara (37% contaminated, with 7,1% incidence of associated infections). Although the preservatives included in anhydrous mascara are stable for a reasonable length of time, use and subsequent exposure to Staphylococci or Candida albicans allows them to support the growth of Pseudomonas (51,52). The use of refill kits, retaining the used brush applicator, and the addition of non-sterile water, also tend to

increase the risk of contamination (53). Inoculation into the eye occurs with accidental injury to the cornea with the applicator, often with disastrous consequences (53,54). The use of ocular cosmetics, and in particular mascara, should thus be avoided prior to and following ocular surgery. This mechanism is also to be suspected in some cases of chronic eye infections, such as blepharitis (55).

5. other eye infections : superinfection with Pseudomonas may occur when an epithelial defect is caused by infection with another microorganism. This has been demonstrated in cases of herpes simplex keratitis (56). Other contributing factors were implicated however, such as concomitant corticosteroid use. Burns reported a case following the use of a topical steroid preparation for chronic herpetic keratitis (14). The drops were prepared in a hospital pharmacy, and were found to be contaminated with Pseudomonas aeruginosa.

1.2.3.2 The tear film

The tear film plays both a physical and a direct antibacterial role in the defence of the ocular surface. In addition its presence is necessary for the proper function of the corneal epithelium and the conjunctiva.

Tear flow across the ocular surface is maintained by reflex blinking and tear drainage via the nasal puncta. This continual flow washes away any debris on the surface of the eye, such as foreign bodies, desquamated epithelial cells, and exhausted tear components (57). Any irritation of the surface causes copious reflex tearing and blinking. This serves to dilute and remove the causative agent.

The tears also contain various factors with a known antibacterial action (adapted from Lemp et al.)(57):

Factor	Action
lysozyme	bacterial cell wall lysis
lactoferrin	antimetabolite action by iron sequestration
beta lysin	cell membrane rupture
secretory IgA	prevention of bacterial adherence
IgG	promotion of phagocytosis / complement-mediated cell lysis
complement	bacterial cell lysis

The secretory IgA system (sIgA) may be considered the first line of defence in the immune system of the eye. IgA is synthesized in the submucosal plasma cells between the lacrimal glands and the conjunctiva, the secretory component being added during its passage through the epithelium (58). In contrast to the direct antibacterial action of IgG, sIgA is believed to act primarily by coating the invading organism and thus inhibiting its adherence to the ocular surface. McClellan et al. (59) have found average levels of 17mg% IgA in tears, with a similar (14mg%) average content of IgG. The submucosal tissue of the conjunctiva also contain mast cells, which release vasoactive substances in response to inflammation or injury (58). The resultant transudate of serum contents boosts tear immunoglobulin levels, particularly of IgG and IgE (59). Both the classic and alternate pathways of the complement system have been detected in human tears from normal subjects (60), and can thus be include in the normal ocular defence mechanism. Activation of the system by various factors, such as bacterial LPS, Ig aggregates, or Ab-Ag complexes, results in bacterial cell membrane

lysis, PMN chemotaxis, histamine release, opsonization, and viral neutralization.

If for any possible reason tear volume is deficient, this protective mechanism breaks down, the level of antibacterial substances is diminished, and erosion of the epithelium may lead to infection by pathogenic bacteria. In patients with 'dry eye' the levels of lactoferrin and lysozyme were shown to be significantly depressed. IgG levels were significantly elevated, suggesting a higher incidence of subclinical infections (61) The nutritional state of the individual may affect this system. Apart from its general effect on immunocompetence via depression of cell-mediated immunity and neutrophil bactericidal efficacy, protein-energy malnutrition is known to affect complement, the sIgA system, and to lower lysozyme levels (62). Hypovitaminosis A is common feature among malnourished Third World populations. This results in xerophthalmia and subsequent erosion of the corneal epithelium. An analysis of 100 such cases revealed 29 with ulceration, 22 perforated eyes (8 due to Pseudomonas), frank pathogens in 46,

and potential pathogens in 4 (63). Inoculation of vitamin A deficient rabbits by instillation of Pseudomonas into the conjunctival sac produced ulceration without any additional trauma to the cornea (64).

Breakdown of the tear film also occurs with any defect in the normal apposition of the eyelids and the globe. This is a feature of senile ectropion and exocrine exophthalmos. Inability to close the lids may occur in Bell's palsy, coma, or because of injury to the lids themselves (57,65). The proper nursing care of the comatose patient is a particular problem, especially if the underlying condition predisposes to colonization with Pseudomonas. An example of this is seen in a report of 2 cases, both of which had a tracheotomy performed, 1 of which was receiving steroid therapy (8). Another report lists 6 cases of Pseudomonas keratitis resulting from careless intubation technique with obtunded patients who had infected sputum (66). Mechanically ventilated patients are a classic risk group (67). Various protective techniques are available, eg., steriostrips, eye

patches. Antibiotic-coated scleral contact lenses are useful in neurosurgical cases where the need to observe pupillary reflexes precludes the use of tarsorraphy. This system was used to good effect by the US Medical Corps in Viet Nam (68).

1.2.3.3 Corneal tissue and systemic factors

The corneal stroma is a transparent tissue and is thus , of necessity, avascular. This, and the absence of lymphatic vessels, makes it immunologically weak. IgG has however been detected in the rabbit cornea (69). It was shown to be present throughout the corneal stroma, but not in the epithelium, Descemet's membrane, or the endothelium. The highest concentrations were found in those corneal lamellae just beneath the epithelium. Studies with haemoglobin have shown that substances with a molecular mass up to 500 000 can diffuse within the corneal stroma (70). IgG, with a MM of 140 000, would thus be expected to penetrate corneal tissue. Similarly, complement components C1, C4, C2, C3, C5, C6, and C7 have been detected in the cornea (71). When compared with normal serum levels of these components, it was

found that those with the lowest MM, C2 and C7, attained the highest relative levels in the cornea. The importance of complement in the ocular defence mechanism has been demonstrated in mice (72). Depletion of C3, by intraperitoneal injection of cobra venom factor, increased the susceptibility to Pseudomonas keratitis of normally resistant strains of mice. Instead of clearing in 4-6 weeks as usual, the majority of infected eyes perforated. Depletion of C5 did not have the same effect, indicating that full lytic C' activity is not necessary, only the phagocytic action mediated by C3 activation. Other factors are involved though, as the BALB/c strain, which has normal C3 levels, was shown to be susceptible. Depletion of C3 has recently been shown to decrease the extent of the PMN response to Pseudomonas invasion (73).

The major host response to Pseudomonas corneal infection is local infiltration of polymorphonuclear leucocytes (PMN's). C3-C5 cleavage products are largely responsible for the neutrophil chemotaxis, immune adherence and metabolic activations leading to this infiltration.

An investigation of the opsonophagocytosis of Pseudomonas by human PMN's showed that IgG natural antibodies, properdin, and C3 proactivator were necessary for this to occur (74). The addition of immune IgG eliminated the need for the last two factors. Only C4 could be detected in the aqueous humour of rabbits and monkeys, even after intraocular injection of Newcastle disease virus (75). This can be explained by the presence of tight junctions between the endothelial cells of the capillaries under the ciliary epithelium, preventing the egress of high MM proteins such as the C' components (76).

The immunocompetence of the cornea itself is thus largely dependent on the immunological state of the host as a whole. Any alteration in the various components of the immune system will be reflected to some extent in the cornea. The major serum antibodies active against Pseudomonas appear to be opsonizing antibodies of the IgG and IgM classes (77). The role of cell-mediated immunity in the defence against Pseudomonas is less clear, although various defects of T-lymphocyte function can

increase susceptibility (6). The reticuloendothelial system (RES) of mononuclear phagocytic cells is important in the host response to Pseudomonas bacteraemia (78).

Systemic immune-deficiency states due to the following causes have an effect on ocular defences (79):

1. steroid therapy, chemotherapy, immunosuppressive therapy
2. leukaemia
3. malnutrition
4. congenital immune-deficiency states
5. diabetes mellitus
6. burns, crush injuries

Various case reports illustrate these points. Mody (79) reported 2 cases of Pseudomonas keratitis, one due to aggranulocytosis induced by prolonged systemic administration of choramphenicol, the other in a patient with extensive burns. Topical use of corticosteroids for chronic inflammatory conditions can also predispose to Pseudomonas corneal infection (80). The influence of these factors is also seen in Pseudomonas infections of

other tissues. The following survival statistics, with respect to various treatment regimes, were noted in 52 patients at Memorial Sloan-Kettering Cancer Center, who developed Pseudomonas bacteraemia (81):

Treatment (prior to sepsis)	Survival (%)	
	Controls	Treated
antineoplastics	6/13 (46)	10/39 (26)
radiation	22/13 (15)	14/39 (36)
steroids (pre-sepsis)	12/26 (46)	4/26 (15)
antibiotics (pre-sepsis)	6/10 (60)	10/42 (24)

This graphically illustrates the considerable risk presented by iatrogenic Pseudomonas infections in already debilitated patients. An outbreak of Pseudomonas urinary tract infection (UTI) was reported in a leukaemic ward in Dublin, Eire. A common R-plasmid, conferring multi-drug resistance, was found in 5 cases on the ward (82). A study of 38 cases of Pseudomonas bacteraemia showed that a better prognosis could be correlated with higher serum antibody levels, and with a lower prevalence of leukaemia and immunosuppressive therapy (77). Congenital hypogammaglobulinaemia (with no defects of cellular immunity) has been shown to increase susceptibility to Pseudomonas bacteraemia (83). The same could be expected with Pseudomonas

keratitis. Certain cases have been attributed to the relative immuno-incompetence of the pre-term infant and neonate (84-86). Faecal contamination may have been a contributing factor in these cases. A study of 117 neonates found 44 with Pseudomonas colonization, of which 3 had conjunctivitis (87). No other local or systemic infections were noted. Faecal carriage rates of up to 56,6% have been recorded in hospital-born infants, compared to a mean of 17,1% for healthy adults (88). The authors also measured faecal carriage in home-born infants, and found an incidence of only 6,0%. This they attributed to more prevalent and earlier established breast-feeding at home, as well as the lack of contact with other, possibly colonized, infants. This is supported by the fact that a direct relationship between maternal and neonatal anti-LPS antibody levels has been demonstrated by workers at the University of Natal Medical School (89,90). They also found that these antibodies could be transferred in the colostrum.

1.2.4 Pathogenetic factors of Pseudomonas aeruginosa

The relative paucity of pathogenicity factors possessed by Pseudomonas has been quoted by Bergan as a reason for its low virulence (91). Bergan also differentiated between those factors associated with an enhanced tendency towards colonization, and those with a direct toxic action. Only those factors which have a direct influence on Pseudomonas invasion and infection of the eye will be dealt with here.

1.2.4.1 Pili

Pili are long, thin appendages arising from the surface of the bacterial cell. They are hydrophobic, and therefore "sticky" in nature (92). As has been discussed earlier (vide 1.2.3.1), bacterial pili (fimbriae) are thought to be involved in the adherence of Pseudomonas to host cells, in particular to exposed corneal stromal cells. It has also been suggested that they exert an antiphagocytic effect, as has been demonstrated in the case of Neisseria gonorrhoea (93). Flagellar antigen preparations have been

isolated, and antisera produced against them. Mice immunized with these flagellar antigens (FAg) showed increased resistance when burned and challenged with Pseudomonas, due to immobilization of the organisms in the burned skin tissue (95).

1.2.4.2 Lipopolysaccharide

The LPS of Pseudomonas is the immunological target of the therapeutic agent investigated in this study. Although the LPS of Pseudomonas is commonly thought to be of much lower virulence than the classical LPS associated with enterobacteria (92), this view has been challenged (96). Although Greer and Milazzo aggreed that that its potency is less than that of the LPS of E. coli, they claimed that it was still significant. They stressed the anticomplementary action of the toxin (96).

Ocular effects have been attributed to the LPS of various Gram-negative bacteria. Howes et al. (97) showed that IV administration of E.coli LPS led to increased vascular permeability, primarily in the ciliary processes. This led to leakage and hence

oedema. A Schwarzmann-type reaction can also be elicited in the eye. The LPS of E.coli, Shigella flexneri, Serratia marcescens, and Salmonella typhi were shown to act in this manner (98). These workers called this an "endotoxic ophthalmopathy", characterised by dacryoadenitis, oedema of the eye socket, exophthalmos, atrophy and shrinkage of the eye, opaque pupils, and ulcerative keratitis. Similar studies with Shigella flexneri LPS noted signs of increased vascular permeability, with disruption of the blood-aquaeous barrier, attributed to e localized Schwartzman phenomenon (99). Shimuzu described a similar reaction with the same LPS, resulting in hyperaemia conjuncticalis, epiphora, discharge, exophthalmos, enophthalmos, phthisis bulbi, corneal ulcer, dilation of the iris, and iritis (100). Intra-corneal injections of E.coli LPS were shown to result in keratitis and vascular scarring in 2-3 weeks. Vascular permeability changes were noted, resulting in limbal aggregation of PMN's and protein extravasation (101). Intravitreal and suprachoroidal injections of E.coli extracts caused

lenticular opacity, possibly mediated via the production of uveitis (102). Intravitreal injections of Shigella LPS caused PMN infiltration, miosis, moderately intense dilation of the iris, conjunctival and limbal vessels, and breakdown of the blood-aqueous and blood-vitreous barriers (103). Exposure of the eye to LPS can therefore be seen to have a variety of results. Marked variations between animals, and also between contralateral eyes, have been noted. LPS effects in the eye are thought to involve the prostaglandin system, since PG-synthetase inhibitors such as indomethacin or acetylsalicylic acid can inhibit the ocular effects of both Shigella (104), and E.coli LPS (105).

Pseudomonas LPS has been implicated in the formation of corneal rings. The injection of viable Pseudomonas cells, as well as of heat-inactivated suspensions of Pseudomonas and E.coli (but not Gram-positive bacteria), causes the formation of such corneal rings (106). These were shown to be accumulations of PMN's. Immunofluorescence studies revealed C3 and

properdin, but no immunoglobulins, indicating that LPS activation of the alternative pathway of complement was responsible. This mechanism is also suspected in the aetiology of non-infectious ring-shaped keratitis, commonly associated with contact lens wearers whose storage containers and solutions are contaminated with Pseudomonas (107).

1.2.4.3 Exoenzymes

Alkaline protease, produced by Pseudomonas aeruginosa, was isolated by Morihara in 1963 (108). Depending on the method used, MM's of 12000, 21000, and 33000 have been recorded (109). In vitro, various nutritional requirements have been demonstrated for protease production (110,111). The production of protease has been correlated with the expression of virulence by Pseudomonas (112). Clinical isolates showed a predominance of protease production (98%) when compared to natural soil/water isolates (54%). The severity of the infection has also been shown to depend on proteolytic activity. Isolates from corneal tissue had extremely high levels of proteolytic activity, producing an average zone of inhibition on skim

milk plates of 5,2mm, compared to 3,8mm for all facial isolates. Protease production has also been shown to correlate with the degree of invasiveness in cystic fibrosis cases (113). Protease was found to be necessary for the full expression of virulence in a burned mouse model (114,115), and in a mouse eye model (116). Its effect in various disease states has been reviewed by Wretlind and Pavlovskis (117).

Intracorneal injections of submicrogram quantities of purified proteases have been found to cause gross corneal damage similar to that seen during infection with viable Pseudomonas aeruginosa (108). Light and electron microscopy revealed a combination of acute inflammation and liquefaction necrosis, i.e., degeneration and necrosis of the epithelium, endothelium and keratocytes; infiltration, degeneration and necrosis of PMN's; destruction of the stromal proteoglycan ground substance; dispersal of structurally normal collagen fibrils; accumulation of plasma proteins and fibrin in the nectrotic corneas. Less purulent discharge and PMN infiltration was noted than that

which occurs with viable cell infections. Protease production is therefore not instrumental in the chemotactic process. These observations support the theory that Pseudomonas protease induces severe corneal damage by causing the loss of proteoglycan ground substance, thus resulting in the dispersal of undamaged collagen fibrils. This weakens the corneal stroma, and leads to descemetocele formation and the ultimate perforation of the cornea by the anterior chamber pressure.

In contrast to the above mentioned proteases, elastases are of limited importance, and have been referred to as a "virulence enhancing factor" (117,118). However, corneal damage can be elicited by purified extracts of the enzyme (119). Elastase is not required for the expression of virulence with respect to corneal invasion and infection (120). However, both enzymes have been shown to reduce bacterial killing by inactivation of complement components (121). No effect on phagocyte function could be detected (both PMN and macrophage). Recent work has shown that both enzymes can inhibit neutrophil chemotaxis, and this could affect the phagocytic defence system by this

mechanism (122).

Production of exoenzyme S has been demonstrated in 38% of clinical isolates (123). The mortality rate associated with strains producing both exotoxin A and exoenzyme S, which have similar mechanisms of action, was shown to be greater than that in patients infected with strains producing either toxin alone. Its effect in ocular infections has not been determined.

1.2.4.4 Exotoxin A

The production of this extracellular toxin was reported by Liu in 1966 (111). Production has been demonstrated in 80% of clinical isolates (123). Quantitative measurements of membrane-bound and extracellular levels have been performed (124). This is the most toxic component of the Pseudomonas pathogenetic system, more than 10 000 times as toxic (by mass) than the LPS produced by this organism, when assayed by the mouse i.p. model (125). It exerts a protein synthesis inhibitory effect, similar to that of the diphtheria toxin (126). It is produced in the form of a proenzyme,

activated by alteration in its covalent structure (127). The toxin is taken up by the host cell by means of receptor-mediated endocytosis, a process which requires calcium (128). The large-scale production of this toxin in chemically defined media (129), and its easy purification and detection (130-132), have enabled extensive studies of its effects to be made. Its inactivation by proteases at first cast doubt on the importance of its role in human infections, but this effect has been shown to occur only at high enzyme concentrations (133,134).

Exotoxin is necessary, together with protease, for the full expression of virulence by Pseudomonas aeruginosa, both in burns (114,115,135), and in cystic fibrosis (136). Its role in various Pseudomonas-induced infections has been reviewed by Young (137), and by Wretlind and Pavlovskis (117). Ohman et al. (120) showed that exotoxin is not required to initiate corneal infections, but does play a role in the persistance of this infection. They suggested a possible inhibition of host bacterial clearance systems, but this is unlikely,

as no effect on complement or phagocytic cells has been detected (121). Corneal tissue damage, as noted by Iglewski et al. (138), is the likely mechanism. Hazlett et al. (139) noted similar ocular effects caused by exotoxin A and by corneal infection with viable cells, except that less purulent discharge and PMN infiltration was seen in the toxin-inoculated eyes. The toxin was also shown to induce cataract.

1.2.4.5 Other factors

Various characteristics of Pseudomonas aeruginosa increase its viablity as a human pathogen.

Pseudomonas has suprisingly simple nutritional requirements, and therefore a variety of substances can support its growth. Contamination of non-sterile medicaments and foodstuffs in the hospital environment can predispose to nosocomial outbreaks (140,141). A study at 7 Spanish hospitals f found that 82% of hospital food samples contained Pseudomonas, as did 4% of non-sterile medicaments (142). Contaminating Pseudomonas are frequently resistant to the antibiotics commonly used in any particular hospital. The above

isolates were resistant to 11/16 antibiotics tested. All could also resist 1000mg/ml chlorhexidine. Tap water is a common source of contamination, especially in hospital pharmacy manufacturing areas. Contamination of diluted disinfectants can occur in this way (143). Pseudomonas is generally resistant to commonly used disinfectants and antiseptics (16), except at the highest concentrations.

Although not generally regarded as a pathogenicity factor per se, the prevalence of antibiotic resistance among clinical isolates of Pseudomonas does contribute to its ability to infect, especially amongst hospital patients. In this regard, the widespread misuse of potent, broad-spectrum antibiotics is apressing problem (143). The clinical relevance of antibiotic resistance will be dealt with in more detail later (vide 1.3.2).

1.3 Treatment of Pseudomonas keratitis

1.3.1 Diagnosis and general principles of management

It has long been recognised that, in the absence of treatment, a Pseudomonas corneal ulcer will usually perforate (144). Immediate initiation of aggressive antibacterial therapy is therefore necessary if sight is to be preserved . Delay of even a few hours, or inappropriate therapy, can result in endophthalmitis and loss of the eye (146). Jones has defined a 5 step management routine, as follows (147):

1. clinical diagnosis
2. laboratory procedures
3. initiation of therapy
4. modification of initial therapy
5. termination of therapy.

Clinical diagnosis is based on examination and patient history. The gross symptoms usually encountered are not exclusive to Pseudomonas keratitis. However, the following have been found to be generally indicative of an infective cause

(146):

1. development of white/yellow spot in the cornea
2. severely reduced vision
3. pain
4. lacrimation
5. photophobia

The location of the ulcer is no longer considered useful, as Pseudomonas can cause both central and peripheral ulcers (1). The patient history should also include the following (148):

1. when and how the disease began
2. possibility of ocular trauma
3. exposure to or previous attacks of similar disease and treatment thereof
4. occupation
5. recent travel
6. predisposing factors, such as diabetes; alcoholism; immunologic or allergic afflictions, use of corticosteroids or antimetabolites

No clinical examination can provide a definitive diagnosis, and the initiation of effective and appropriate therapy is further hampered by the fact

that laboratory screening techniques are time-consuming and often non-specific, eg., Gram stain, LAL test. A recent innovation is the use of ultraviolet light fluorescence for the early detection of Pseudomonas eye infections (149). However, a broad-spectrum high-dose antibacterial regimen is usually initiated, and later modified according to laboratory findings (bacterial identification and antibiotic sensitivity tests). The use of steroids is generally contraindicated until a diagnosis has been clearly established (76).

1.3.2 Antibiotic therapy

Two major groups of antibiotics display significant antipseudomonal activity, i.e., the aminoglycosides and the broad-spectrum beta-lactams. Various combinations and other minor groups of antimicrobials have also been used.

1.3.2.1 Aminoglycosides

The following members of this group have been introduced for the treatment of Pseudomonas infections (150):

1. gentamicin
2. tobramycin
3. amikacin
4. dibekacin
5. 5-episisomicin
6. netilmicin
7. sisomicin

Of these, only the first two, gentamicin and tobramycin, are in general use in ophthalmology. Various exotic derivatives have been developed, such as BB-K8, but have a limited usefulness. For example, BB-K8 does not attain sufficient levels in the aqueous after topical, IM or IV administration, and has only proved effective via the intravitreal route, for the treatment of Pseudomonas endophthalmitis (151). Amikacin is seldom used, but its efficacy has been demonstrated in a case of gentamicin-tobramycin resistant Pseudomonas keratitis in a nosocomial infection of a burn patient (152).

The efficacy of gentamicin and tobramycin has been established in several in vitro, in vivo, and clinical trials (153-164). Although tobramycin is more effective than gentamicin in vitro, the two are equivalent clinically (1,158). Differences do exist in respect to the toxicity of the aminoglycoside antibiotics, although all may induce nephrotoxic and ototoxic effects (164-169). In general, they are all irritant on injection and painful when instilled into the eye (165).

The major problem with the aminoglycosides is the rapid emergence of drug-resistant strains of the bacteria, especially in hospitals where such antibiotics are abused. A graphic example of this was provided by a study at the All India Institute of Medical Sciences, New Delhi, India (10). In 1977, 80% of all Pseudomonas clinical isolates at this centre were sensitive to gentamicin. By 1980 only 32% were sensitive. Most of the resistant isolates were obtained from corneal ulcers. technique, and the subsequent emergence of resistant strains and increased incidence of opportunistic infections, has been well documented

(8,177). The mechanisms of pseudomonal resistance to aminoglycosides have been comprehensively reviewed by Bryan (176). More attention is now being given to the role of R-plasmids, especially in the emergence of multi-drug resistant forms (173,178).

1.3.2.2. Beta-lactams

The following beta-lactam antibiotics have been shown to exhibit antipseudomonal activity in vitro (150):

1. Penicillins : carbenicillin
   ticarcillin
   piperacillin
   sulbenicillin
   azlocillin
2. Cephalosporins: cefoperazone
   cefotaxime
   cefsulodin
   ceftazidime
   moxalactam
   Ro 13-9904

3. Thienamycins: thienamycin

n-formimidoyl thienamycin

The efficacy of carbenicillin and ticarcillin has been demonstrated in rabbit models of Pseudomonas keratitis (179-180). Ticarcillin has been used successfully, in combination with tobramycin, in 2 clinical cases (181,182). However, most experience with these newer beta-lactams is only at the in vitro stage (183-188). So far, the most promising appear to be the thienamycin derivatives (189-191).

Resistance to the beta-lactams, usually by plasmid-mediated beta-lactamase production, has also been widely reported (192-196). An ever present problem with any penicillin-type drug is the risk of a hypersensitivity reaction. The reported occurrence of such reactions varies considerably, but remains significant in that a life-threatening anaphylactic reaction may occur (197). Carbenicillin and ticarcillin are also irritant, causing inflammation and thrombophlebitis after parenteral administration, due to platelet aggregation and subsequent bleeding (198). The cephalosporins have irritant properties, as well as being slightly nephrotoxic (198).

1.3.2.3 Combination therapy

Numerous workers have shown synergistic effects when an aminoglycoside and a beta-lactam have been combined against Pseudomonas in vitro (199-209). The use of such combinations, to reduce the incidence of adverse reactions and limit the emergence of resistant strains, has been proposed (210). A combination of ticarcillin and tobramycin has been used in 2 cases of Pseudomonas keratitis, with good results (181,182). A high-dose combination of cephazolin and gentamicin was recommended by Chadhauri and Godfrey (211), based aeruginosa. One was treated successfully with this regimen, the other was lost.

1.3.2.4 Other antibiotics

Other antibiotics tried in vitro and in vivo include nalidixic acid (212), MK-0366 (a nalidixic acid derivative) (213), and polymixin B (145,214).

In a review of antibiotic therapy development with respect to Pseudomonas bacteraemia, Andriole (215) noted that, despite recent advances, the prognosis

had not improved substantially, thus indicating a need for alternative forms of therapy. Although major advances have been made in the development of new antipseudomonal antibiotics for ocular infections, their usefulness is limited by the ability of the organism to rapidly become resistant to the new drug.

1.3.4 Alternative forms of treatment

A number of alternative therapeutic agents have been proposed, including:

1. argon laser phototherapy (216)
2. sugar solutions (217)
3. haparin (218)
4. chelating agents (219)
5. cryotherapy (220,221)
6. cyanoacrylate glue (222)
7. bandage contact lenses, as drug delivery systems (44,223)

The ultimate form of therapy is of course keratoplasty (224), but this is a severe, high-risk procedure. Sufficient healthy tissue must be preserved in order to support a graft, and failure of treatment often precludes this means of

management. A promising new approach is appears to be the use of immunotherapy.

1.4 Immunotherapy for Pseudomonas infections

The failure of current conventional therapy has provided the impetus for increased activity in the field of immunotherapy (225-227). This type of therapy depends on the reaction between the various antigenic components of the organism, which are involved in the pathogenesis of the disease state, and antibodies directed against them. Both active and passive forms of immunotherapy have been used in the past. Early research in this field was largely empirical, in that non-specific preparations were used, eg., in 1957 Rosenthal et al. (228) used a human gamma globulin preparation to protect mice against intraperitoneal challenge with Pseudomonas aeruginosa. In 1958 Fisher and Manning (229) found that a poliomyelitis IgG preparation protected mice against a similar challenge with many Gram-negative bacteria, including Pseudomonas. In 1961 Fisher (230) showed that pooled human gamma globulin had antibacterial properties, and that it was particularly rich in

antibodies to Pseudomonas aeruginosa and Staphylococcus aereus. In 1962 Waisbren and Lepley (231) used gamma globulins as an adjunct to conventional antibiotic therapy for Pseudomonas bacteraemia. In 1964 Feller et al. (232) reported the successful use of a killed-cell vaccine to raise antibodies against Pseudomonas. In 1965 Feingold and Oski (233) used plasma from a Pseudomonas sepsis survivor (following extensive burns) to treat a Pseudomonas infection in another, immunocompromised, patient.

Subsequent work has involved the use of antibodies prepared against specific antigens.

1.4.1 Exoenzyme preparations

Pseudomonas protease and elastase toxoids have been shown to protect against subsequent corneal damage in the mouse, following inoculation into the cornea of the homologous enzyme (234). These workers found that an antiprotease antiserum was also protective, but that anti-elastase had no effect. This was confirmed by Cryz et al. (235) in a murine burn wound model. Synergy was demonstrated between a kanamycin derivative, DKB, and various vaccine and antisera combinations directed at

Pseudomonas protease, elastase and OEP ("original endotoxic protein") (234). Similar vaccines were later shown by Homma (236) to confer protection against haemorrhagic pneumonia in mink. The same group of workers (237) elicited IgE production in mice with a formol toxoid of Pseudomonas elastase. Antibodies to both exoenzymes have been demonstrated in man (238), and it is thought that these are the result of asymptomatic Pseudomonas colonization in childhood.

1.4.2 Exotoxin A preparations

Pavlovskis et al (239) used an antitoxin preparation in a burned mouse model. Immunization with antitoxin A serum 20 hours prior to Pseudomonas inoculation enhanced survival and reduced bacterial counts in the blood and liver, but only when a toxigenic, low protease producing strain was used. Prolonged survival was attained when the mice were infected with a toxigenic, high protease producing strain, but no effect was seen when a non-toxigenic inoculum was used. The antiserum produced had no bactericidal, antiprotease or anti-LPS properties. Antibodies to exotoxin A have been detected in children aged 1 to

4 (238). High titres of antitoxin A antibodies at the onset of Pseudomonas septicaemia in man were found to correlate with a good prognosis (240). As with anti-elastase sera, anti-exotoxin A antisera had no effect in a murine burn wound model of Pseudomonas infection (235).

1.4.3 Extracellular slime preparations

Immunization of rabbits with a Pseudomonas slime extract has been shown to protect against corneal damage following inoculation with mucoid, proteolytic strains of Pseudomonas (241) Serum from rabbits with high "antislime haemagglutin" levels was able to transfer this protection to mice, indicating that humoral immunity mechanisms were involved. Although Pseudomonas is normally resistant to phagocytosis by PMN leucocytes, antibodies to slime glycolipoproteins opsonized these bacteria, and protected against viable cell challenge in experimental infections (242). The opsonization was most effective in the presence of complement. However, this antiserum had no bactericidal properties, alone or with complement.

1.4.4 Lipopolysaccharide preparations

The possibility of using LPS as the antigenic target was demonstrated by Freedman (243). He was able to prevent the pyrogenic effects of endotoxin in mice by means of injections of rabbit antiserum. The mice showed a resistance to endotoxin isolated from different bacterial species, and also to Gram-negative infection. This was confirmed by Davis et al. (244). They found that homologous rat antisera caused lethal anaphylactic reactions in mice. This they suggested was due to antibodies to the 'O' antigen, the protective antibodies being directed against the core antigens of LPS. Crude Pseudomonas antigen preparations were used by Johnston and Syeklocha (245) to raise anti-LPS antibodies in rabbits. These antisera protected mice against IV challenge with Pseudomonas, but were type-specific for the PA-7 strain. The authors considered LPS to be the target antigen.

Various vaccines containing a number of Pseudomonas cell wall antigens have been developed. Parke-Davis Laboratories have produced a heptavalent vaccine, known as "Pseudogen". The development of this vaccine, and its use in

volunteers to raise anti-Pseudomonas antisera, has been reviewed by Fisher (246). No trials have been conducted using the product to treat or prevent Pseudomonas keratitis. A significant incidence of toxicity was noted when this vaccine was used to protect against bacteraemia in patients undergoing antineoplastic therapy, occuring in 92% of patients vaccinated (247).

Wellcome Research Laboratories, in conjunction with the Birmingham Accident Hospital Burn Unit, have produced a 16 component vaccine, known as PEV-01 (248). Use of this vaccine, or of human antisera to this vaccine raised in healthy volunteers, has largely been restricted to burn cases. Trials in Birmingham and at Safdarjang Hospital, New Delhi, have demonstrated both efficacy and safety (249,250). However, a major drawback has been its specificity for Pseudomonas. Moreover, this vaccine did not prove effective in a double blind placebo controlled study presently conducted in Durban on children with large burns (Mickel, pers. comm.) The latest report from this group showed that Klebsiella pneumoniae had emerged as the

(pers. comm. Prof R Mickel, Dept. of Paediatric Surgery, University of Natal Medical School, Durban, South Africa)

dominant Gram-negative species causing bacteraemia in burn patients at Safdarjang Hospital (251).

IgG preparations, modified to allow IV administration, have been used in a number of trials. Cutter Laboratories' MISG was shown to be effective against 6/7 Pseudomonas immunotypes in a mouse i.p. model (252). The same product was shown to contain anti-LPS antibodies, and to be bactericidal in combination with complement (253). Effective prophylaxis with this preparation was achieved in a murine burn wound sepsis model.

Pollack and Young (240) were able to show a correlation between a good prognosis in cases of Pseudomonas septicaemia, and the levels of anti-LPS and anti-exotoxin A antibodies at the onset of the infection. Pollack also examined the anti-Pseudomonas activity of 7 different types of IGIV preaparations, including one from Parke-Davis produced by vaccination of healthy volunteers with "Pseudogen" (254). All were shown to contain Pseudomonas-specific anti-LPS and anti-exotoxin A antibodies, to support the in vitro killing of

Pseudomonas by human PMN's, and to protect mice against a fatal burn wound infection. This protection was also shown to be dose-dependent. Higher doses were needed to protect granulocytopaenic mice. A higher antibody titre was seen in plasma samples collected in Mexico than those from USA-derived products. This was seen as supporting the view that serum antibodies specifically directed against Pseudomonas can be expected to be present in populations exposed to the bacteria, as determined by socio-economic, environmental, and geographic factors (254).

A recent report has confirmed the importance of anti-LPS antibodies in combating infections caused by Pseudomonas. Anti-LPS, but not anti-elastase or anti-exotoxin A, antibodies were effective, both therapeutically and prophylactically, in a murine burn wound sepsis model (235). Bacterial multiplication on the skin was not affected, but bacteraemia and hepatic infection were prevented. The antibodies used were however serotype-specific.

The Braude group in San Diego, California, have

developed a method of raising antibodies, directed at the core glycolipid of Gram-negative bacterial LPS, which have a broad-spectrum activity. The E.coli J5 mutant is deficient in uridine diphosphate galactose, and consequently produces an endotoxin without oligo- and polysaccharides where the 'O' antigen resides. Vaccination with killed cells of this E.coli results in high serum concentrations of anti-LPS antibodies effective against Pseudomonas aeruginosa (78). The 'O' antigen is not the target of this antiserum, hence its broad-spectrum nature. Complement is apparently not involved in the bactericidal action of these antibodies, which depend on opsonization of the bacteria to aid removal by PMN's or the RES (255).

Monoclonal antibodies, directed against the 'O' antigen of particular Pseudomonas serotype's LPS, have been produced (256). Their excessive specificity, and the large number of Pseudomonas serotypes appears to make this avenue of attack impractical for the time being.

1.5 The development of Anti-LPS

Gaffin and colleagues have devloped a method of detecting high concentrations of anti-LPS IgG antibodies in donor serum samples. This was first used, in Israel, to protect cats against experimental haemorrhagic shock (257). A rapid ELISA procedure now enables large quantities of such antiserum to be isolated by the Natal Blood Transfusion Service (258,259). In addition, a vaccine has been perfected, which is used to raise anti-LPS antibodies in horses. These are harvested by plasmapheresis, at Summerveld Stables, Shongweni. This equine hyperimmune antilipopolysaccharide plasma (Anti-LPS) has been used in this project.

Previous and concurrent work with Anti-LPS has included the successful treatment/prophylaxis of :

1. gastroenteritis, endotoxaemia, and various Gram-negative infections in horses (260)
2. Pseudomonas peritoneal infection in mice
3. E.coli septic abortion in rats (261)
4. Pseudomonas burn wound sepsis in guinea pigs (262).

5. X-irradiation overdose in mice and rabbits (263)
6. superior mesenteric artery occlusion shock in rabbits (264)

Prior use in veterinary practice has shown that topical administration of Anti-LPS has a rapid bactericidal action against a wide range of Gram-negative bacteria . For example, lavage of the uterus with plasma effectively treated several intra-uterine Klebsiella infections in mares. It was therefore decided to apply this type of immunotherapy in other Gram-negative surface infections, such as Pseudomonas keratitis. This project was conducted in order to investigate such a possibility.

CHAPTER 2

2. MATERIALS AND METHODS

2.1 Introduction

This section deals with those materials and methods used throughout the project. Specific details of techniques used in one section only are described in the relevant chapter.

2.2 Animals

The following animals were used in the in vivo trials:

1. Rabbits (Oryctolagus cuniculus) - pigmented adult rabbits, of a mixed non-inbred strain, were obtained from the Natal Institute of Immunology, Pinetown. The rabbits were housed individually, under natural lighting conditions, and were fed on rabbit pellets and tap water ad libitum. Those used had a mass of 2,5 to 3,5kg. All were judged to have normal corneae and conjunctivae before use.
2. Guinea pigs (Cavia porcellus) - guinea pigs of a mixed non-inbred strain were obtained from the

same breeding facility. They were housed and fed in the same manner as the rabbits, except that fresh vegetable matter was provided as a source of moisture. Animals with a mass of 450-650g were used.

3. Baboons (Papio ursinus) - adult baboons from the Natal Medical School Animal Colony were used. They were housed individually and maintained on a vegetarian diet. Tap water was available ad libitum. The two animals used had a mass of 6,8 and 8,0kg.

2.3 Pharmaceutical products

2.3.1 Saline - sterile saline was supplied in the form of Sodium Chloride Injection B.P. 0,9% (M/V) (Sabax Laboratories). It was administered directly from a 1000ml Viaflex container, via a Plexitron 15 drop/ml administration set. The saline was stored at 4oC, and warmed to 37oC if intended for ocular administration.

2.3.2 Anaesthetics - the following anaesthetic agents were used:

1. oxybuprocaine hydrochloride (Novesin 0,4% eye

drops; Dispersa) - for local ocular anaesthesia.

2. ketamine (Ketalar 10mg/1ml; Parke-Davis Laboratories) - for general anaesthesia of baboons. The dosage used was 10mg/kg body mass (IM).

3. ether - for general anesthesia of guinea pigs (by inhalation).

2.3.3 Corticosteroid drops - dexamethasone disodium phosphate (Spersadex 0,1% eye drops; Dispersa).

2.4 Microbiological products

2.4.1 Bacterial cultures

Lyophilised cultures of Pseudomonas aeruginosa were obtained from the Department of Microbiology, University of Natal Medical School.

The bacteria were stored as concentrated saline suspensions, at OoC. Nutrient broth cultures were prepared as required.

2.4.2 Media

The following microbiogical media were used:

1. Nutrient broth - prepared by dissolving Bacto-Nutrient Broth dehydrated powder (Difco Laboratories) in distilled water (8g in 1000ml).

It was sterilized by autoclaving at 121oC for 15 minutes and then stored at 4oC.

2. Nutrient agar - prepared by the addition of 1% (M/V) Bacto-Agar to nutrient broth. Dissolution was aided by heating in a microwave oven for 60 seconds. Sterilization was performed as above, and the solution was poured into sterile, disposable petri plates, under laminar flow conditions, and stored at 4oC until required.

2.4.3 Methods

Bacterial cell counts were performed using an improved Neubauer chamber (0,0025mm2).

All manipulations of bacterial cultures were performed in a biohazard quality laminar flow cabinet with vertical air flow. All reasonable precautions were taken to prevent contamination with human pathogens, eg., wearing of gloves, masks; incineration of used materials.

2.5 Anti-LPS plasma

The equine hyperimmune antilipopolysaccharide

plasma (Anti-LPS) used in this project was obtained from Atox Pharmaceutical Co., 14 Old Main Rd, Gillits.

Horses were suitably immunized by a patented method devised by Dr S.L. Gaffin . Antibody-rich plasma was harvested by plasmapheresis, performed at Summerveld Stables, Shongweni. Unit doses of 10ml were packed, under laminar flow conditions, and stored at 0oC. These were warmed to 37oC prior to ocular administration, and given dropwise via a length of Tygon tubing, fitted with a drip tap. Plasma intended for parenteral use was sterilized by filtration through a 0,2um Micropore filter, and stored in sterile, pyrogen-free Falcon tubes, stored at 0oC.

The specific anti-LPS antibody concentration of the plasma was measured by an enzyme-linked immunosorbent assay (ELISA) developed by Gaffin et al (258). Those batches used for this study contained 1000-1500 ug/ml of LPS-precipitible antibodies.

2.6 Glassware, equipment

Wherever possible all equipment was of a sterile, disposable type, eg., pipettes, test tubes, petri plates, hypodermic needles.

All glassware was rendered pyrogen-free by heating at 200oC for 4 hours.

2.7 Statistical methods

The following statistical tests were used for analysis of data collected in this project:

1. chi-square test (four fold table analysis)
2. Student's 't' test
3. Fisher's exact probability test

All calculations were performed on a Hewlett-Packard HP 87 computer. The computer programs used are attached in Appendix A. The 5% level of significance was used throughout.

CHAPTER 3

3. OCULAR TOLERANCE STUDIES

3.1 Introduction

Any investigation of a new therapeutic agent must include examination, by various means, of the safety of such an agent.

Prior observations of the safety of Anti-LPS in various animal species obviated the need for such toxicological experiments as corporal mass change measurements and mortality trials (260, 263,265). Primarily, what was needed was a demonstration of tolerance to various routes of ocular administration, before the efficacy of the product could be tested against experimental infections.

3.2 Materials and methods

3.2.1 Topical administration

3.2.1.1 Rabbits

The eyes of 5 rabbits were used in this study. There were no ocular defects before the trial commenced.

Four rabbits received the following treatment regimen:
left eye - Anti-LPS plasma, 40 drops/minute for 5 minutes (standard plasma dose), 3 times a day
right eye - sterile, normal saline, in the same manner.
One additional rabbit received the same treatment, except that the eyes were alternated daily with respect to which received plasma, and which saline. The trial was conducted over a period of 12 days.

The eyes were examined daily for signs of ocular irritation, eg.,

conjunctival hyperaemia

oedema

tearing

blepharospasm

They were also examined at the slit-lamp biomicroscope.

3.2.1.2 Baboons

Two adult baboons were used in this trial. They were anaesthetized with ketamine IM (10mg/kg body mass), and then plasma was administered topically to the left eye, once every alternate day. The standard plasma dose was used. The trial was conducted over a period of 14 days.

The eyes were examined for gross signs of ocular irritation, prior to each treatment, and from outside the cage on days they did not receive plasma.

3.2.2 Sub-conjunctival administration

Two healthy rabbits, with no ocular defects, were used. The left eyes each received a sub-conjunctival injection of 0,5ml of Anti-LPS daily for 7 days. The right eyes were left as untreated controls. The plasma used was sterilized by filtration (Micropore 0,2um filter), stored in sterile, pyrogen free tubes at 0oC. It was warmed to 37oC prior to use use and given, under local anaesthesia (Novesin 0,4%), using a sterile, disposable U-100 insulin syringe with a 27-gauge

needle.

The eyes were examined daily, prior to each injection, for signs of ocular irritation or corneal pathology. They were examined again on days 14 and 30 of the trial. Examination at the slit lamp was performed on day 14.

3.3 Results

3.3.1 Topical administration

3.3.1.1. Rabbits

No ill-effects were noted, except for slight upper limbal congestion in 2 eyes, 1 of which received plasma, the other saline. At the conclusion of the trial all eyes were shown to be normal by slit-lamp biomicroscopy (in terms of cornea, lens, and fundus oculi).

3.3.1.2 Baboons

In the healthy baboon, the plasma was shown to have no adverse irritant effects, and no signs of inflammation were noted at any stage. The obvious problems of handling such animals prevented

examination at the slit-lamp, but no ocular defects could be seen under loupe examination.

3.3.2 Sub-conjunctival adminstration

Both plasma treated eyes showed slight and transient conjunctival hyperaemia after repeated injection. This was evident on day 2 and 5 in the 2 rabbits. Inflammation was restricted to the injection site only; the palpebral conjunctiva remained clear throughout. On days 14 and 30 both eyes were normal. There was no necrosis or scarring at the injection sites and no visible corneal pathlogy. Biomicroscopy revealed no defects at all. Both control eyes remained clear throughout the trial. In summary: Anti-LPS produced only slight ill effects.

3.4 Discussion

The major antipseudomonal antibiotics are the aminoglycosides. On instillation these usually cause a slight burning sensation (154), although more severe reactions have been recorded. These

include increased irritation, erythema, lid oedema, chemosis, and folliculopapillary reaction (164). They were reported in a study from which all patients with known hypersensitivity to aminoglycosides had been excluded. All patients who had received antimicrobial chemotherapy in the preceding 48 hours were also excluded, so that the reactions noted were not due to prior sensitization. Such reactions have been reported in as many as 10% of patients treated topically with gentamicin, and the medication was withdrawn in 80% of these cases (156). Sub-conjunctival injections of the aminoglycosides can elicit a more serious reaction. Administration of neomycin and soframycin causes extreme pain and irritation, especially after repeated injection (266). Gentamicin and tobramycin are tolerated. Although minimal chemosis and conjunctival injection does occur, this usually disappears after 24 hours. However, scarring can occur after sub-conjunctival injections of gentamicin, and continuous lavage has resulted in systemic toxicity (267). Tobramycin is slightly less irritant, but similar reactions can be expected.

Various animal studies have been conducted to determine the ocular tolerance of particular antibiotics (179,180,212,214,268-271). Determination of tolerance to topical administration usually involves the instillation of the product 3 times a day for a period of 1 week. The eyes are examined daily for the such signs of ocular irritation as conjunctival hyperaemia, chemosis, oedema, tearing, or the production of any discharge or exudate. Tolerance of sub-conjunctival injections usually involves the daily injection of volumes of 0,2 - 0,5ml for a similar period. Some workers have reported a lack of side effects after a single dose (271), but repeated injections are required before any conclusion may be reached. The same parameters are monitored, as well as any signs of scarring, haemorrhage, iris congestion or any corneal pathology. In both cases ophthalmoscopic examinations of the cornea, lens, and fundus oculi should be performed.

In this project ocular tolerance to topical adminstration of Anti-LPS was first examined in the

rabbit. Slight upper limbal congestion was noted in 1 test and 1 control eye. The plasma was therefore considered safe for topical use. The experiment was repeated in the baboon. The problems of handling such animals precluded more frequent administration than once every second day, but the trial was conducted over an extended period. At no stage were any signs of ocular irritation noted.

An initial trial, in which a single 1,0ml sub-conjunctival injection of Anti-LPS was given to one eye of a healthy rabbit while the contralateral eye received the same dose of sterile saline, showed that the plasma was not irritant when administered by this route. However, because of the foreign protein nature of the product, it was thought that repeated injections over a period of time would be needed before any meaningful conclusions could be drawn regarding tolerance of this route of administration. The method used by Furgiuele was adopted (268). The slight conjunctival hyperaemia seen in both treated eyes was transient in nature, and no signs of ocular

irritation could be detected at 2 and 4 weeks after initiation of the trial. Any conjunctival oedema noted was only at the injection site, and was considered to be due to the trauma of the injection and not to the plasma itself. At no stage was there any discharge or opacity present. Biomicroscopy revealed no abnormalities at all. This was consistent with the results obtained by Furgiuele (268) and Purnell and McPherson (272) when testing gentamicin and tobramycin. More severe reactions were noted by Belfort et al. (270), eg., subconjunctival haemorrhages. Nevertheless, these workers found such adverse effects acceptable, since they subsided in 4-6 days and no corneal lesions were noted at all.

Although the side effects encountered with the ocular administration of antipseudomonal antibiotics are seldom so severe as to preclude the use of the agent, the patient is already experiencing considerable discomfort. Any additional insult to the infected eye should be avoided if possible. It is therefore significant that the equine plasma was well tolerated by the

animals tested. This lack of adverse effects can be partially explained by the nature of the plasma, i.e.,

1. it is isotonic with lacrimal secretions
2. it has a pH of about 7,4
3. it was warmed to 37oC before administration

The human eye could be expected to tolerate administration of Anti-LPS as well. Permission for clinical trials of this nature is at present being sought. These would be conducted by the Department of Ophthalmology, University of Natal Medical School at King Edward VIII Hospital, Durban.

CHAPTER 4

4. TREATMENT OF EXPERIMENTAL KERATITIS

4.1 Introduction

The ultimate measurement of an antimicrobial agent's efficacy is the determination of its effect on the outcome of the disease state in the live animal. Of necessity, any new therapeutic agent has to be tested in animal models, on experimental infections, before clinical trials can be considered. Various experimental models have been used to induce Pseudomonas keratitis in laboratory animals.

Three separate trials were performed in order to determine the following:

1. the effect of Anti-LPS on the outcome of experimental Pseudomonas keratitis in rabbits
2. the effect of corticosteroid therapy on this treatment regimen
3. the effect of Anti-LPS in a guinea pig model

The models used in this project were adapted from those reported by previous researchers, as described below.

4.1.1 The experimental animal

The choice of experimental animal is largely determined by the following factors:

1. ease of handling
2. response to the disease being studied, compared to the human situation
3. availabiliy
4. price

Most trials involving Pseudomonas keratitis have used either rabbits, guinea pigs, or mice. Other animals, such as dogs, have been used, but were not considered suitable for this project.

Rabbits and guinea pigs are easy to handle, and their eyes are large enough for gross clinical examination without a slit-lamp, and for photographic recording of the ocular lesions. The clinical course of Pseudomonas keratitis has been

well documented for both animals (272). This enabled clear conclusions to be drawn as regards the outcome of the experimental infections being studied.

4.1.2 The method of inoculation

Pseudomonas aeruginosa cannot penetrate the intact corneal epithelium, and the organism is capable of attaching only to injured corneal stromal cells (19). This fact was clearly demonstrated in a study using rabbits with hypovitaminosis A (64). This condition leads to breakdown of the tear film, and subsequent erosion of the corneal epithelium. Inoculation of a Pseudomonas culture into the conjunctival sac resulted in corneal ulceration in 1/3 rabbits with severe punctate keratitis, 4/7 with xerosis, but in none of those with mild corneal symptoms or in control rabbits. Corneal trauma is therefore needed when inducing experimental keratitis. Two basic methods have been used:

1. a superficial corneal wound is inflicted, and a

culture or suspension of the bacteria is dropped onto the traumatized corneal surface

2. the bacterial inoculum is introduced directly into the corneal stroma in some manner.

The first method corresponds more closely with the clinical situation where a corneal infection is preceded by an injury of some sort, inflicted by a contact lens, finger nail, mascara brush, or irritant foreign body. This can most easily be accomplished by scarification of the cornea using a sterile needle, as used in this project (274). An alternative is abrasion with a toothed chalazion curette, the so-called Cignetti method (267). Here the cup of the curette holds the inoculum, and the organisms are introduced during abrasion of the corneal surface. Earlier workers used cotton-tipped applicators, dipped in the bacterial suspension and rubbed onto the abraded area, to increase the chances of infection (214).

The second method more closely mimics the situation encountered following removal of a penetrating foreign body, or following penetrating surgery. Contaminated sutures have been used to introduce

Pseudomonas directly into the corneal stroma (275). This method was shown to introduce between 1-100 million organisms, when the suture was first soaked in a culture containing 1-100 million organisms/ml (276).Hessburg's method was adapted for use in this project. More recent trials in guinea pigs have used intrastromal injections of microliter amounts of bacterial suspensions, in order to induce Pseudomonas keratitis (277). Particular strains of known virulance have been used in these trials. Subsequent use, by the same group of researchers, of the needle scarification (to more closely mimic the clinical situation), resulted in similar infections provided the same strain of Pseudomonas was used (278). Another group has used a similar method on rabbits (279). Although a very consistent rate of infection was achieved by these workers, this has not been the case in all models. A study in dogs reported a varied clinical response, despite using a highly standardized procedure (280). This involved trephination of a 5,0mm diameter corneal area, superficial keratotomy to a depth of 0,1mm, followed by a 10ul intrastromal injection of a standard inoculum at

the margin of the trephined area. This method is technically very difficult and requires highly specialized equipment, primarily a stereoscopic operating microscope. For this reason it was not considered useful for this project. It is interesting to note that despite the varied response obtained in this dog trial, the authors concluded that this was "an acceptable biologic model for the study of this destructive disease to the eyes of humans and animals". Similarly, while not exactly corresponding to the clinical situation, the two models used in this project give an accurate indication of the effect Anti-LPS based therapy would have in humans.

4.1.3 The treatment regimen

Ocular infections can be treated by the following routes:

1. topical instillation of drops/ointment
2. sub-conjunctival injections
3. systemically
4. combinations of the above

Choice of a route is governed by the

characteristics of the drug being utilised, and by the severity of the infection. For example, polymixin B is nephrotoxic when given systemically, and very irritant if given sub-conjunctivally. This limits the drug to topical administration (214). On the other hand, ticarcillin has to be given by injection (180). In this project it was decided to use the topical route, at the same dosage used in the ocular tolerance trials (vide 2.2), i.e., 40 drops/minute for 5 minutes (approx. 10ml), 3 times a day.

The relative merits of topical and sub-conjunctival administration of antimicrobials have not been conclusively decided. Various antibiotics are tolerated by the sub-conjunctival route (179,180,270,274). Topical administration has proved more effective in certain trials (281,282). Davis et al. (281) claimed that a combination of topical and sub-conjunctival administration was no more effective than topical alone. Leibowitz et al. (282) found no therapeutic effect after sub-conjunctival injection. It was their opinion that any beneficial effect resulting from this mode

of therapy was due to antibiotic reaching the cornea topically, being refluxed through the needle tract. In contrast, Baum and Barza (283) showed that sub-conjunctival and topical use of gentamicin were equally effective in a rabbit model of Pseudomonas keratitis. They evaluated efficacy in terms of surviving organisms per cornea. The sub-conjunctival route was more effective when evaluated at 9 hours post-treatment, but this difference disappeared after 17 hours. The injections were given every 8 hours, the drops every hour. Concomitant topical and sub-conjunctival administration of gentamicin and tobramycin has been used effectively in a number of experimental trials (267,272,284,285). Sub-conjunctival injections were prescribed in a highly standardized model for antimicrobial evaluation developed by Davis and Chandler (277). This route of administration was not used in the Anti-LPS trials, although rabbit eyes were shown to tolerate repeated sub-conjunctival injections of the plasma (vide 2.3). Previous workers have shown that this route is often impractical in animal trials, as the infected eye becomes too friable (278) Excessive leakage from the injection site

would defeat the object of using this route.

Systemic use of antibiotics is usually restricted to the treatment of endophthalmitis (286). Leibowitz et al. (282) could find no effect on corneal bacteriology when various antibiotics were given systemically to rabbits with Pseudomonas keratitis. Davis et al. (278) showed the IM route to be as effective as either topical administration, or a combination of both routes. However, a later report by the same group showed IM tobramycin to be less efective than topical administration in the same guinea pig model. The IM route was no more effective than placebo when tested in rabbits. The beneficial effects noted in guinea pigs may be ascribed to greater bioavailability of the antibiotic in this animal, as the periphery and mid-periphery of the guinea pig cornea are normally heavily vascularized (282,287). Gentamicin has been detected in the rabbit aqueous after IM and IV injections, but only if the blood-aqueous barrier has been altered (268). The equine nature of Anti-LPS plasma precluded its IV use in rabbits or guinea pigs.

The molecular size of the IgG antibodies was considered too large for effective ocular distribution after IM injection. These routes were therefore not examined.

4.1.4 The evaluation method

The ultimate measure of the outcome of an ocular infection is the resultant visual acuity of the patient. This cannot, for obvious reasons, be used in animal experimentation.

Previous workers have used one of two basic methods:

1. bacteriological status
2. clinical scoring methods

Smolin et al. (267) used surface swabs to determine the number of viable Pseudomonas organisms on the cornea at particular times. They also graded the same infections by clinical parameters (size and density of lesion). Correlation between these two mehods was poor. A gentamicin-treated rabbit showed a pathology of grade 4 (out of a possible score of 5) on the third day of treatment, but no bacteria were detected in

either eye. On the fourth day, the corneas were excised and cultured. The right eye grew 37 colonies, but the left remained ostensibly sterile. In contrast, another rabbit grew 7 and 36 colonies from the left and right eyes respectively, when both were scored as grade 1. In subsequent experiments they used surface swabs on the first day only, to show that eyes from different treatment groups had an approximately equal number of organisms on their corneal surfaces prior to treatment (285). Cultures of excised corneas after 3 days showed a significant decrease in the number of viable organisms, but no clinical difference between the treated and control groups could be detected. They suggested that "the clinical course, instead of reflecting the infectious process, reflects in fact the inflammatory response to the necrosis that is caused by the presence of the organisms". Early bacteriological evaluation of Pseudomonas keratitis, as soon as 8 hours after initiating therapy (279), is based on the assumption that prolonged treatment will further reduce levels of viable organisms, and ultimately affect the clinical course of the infection (285).

Baum and Barza stated that "it would be reasonable to believe that administering an antibiotic ... over a longer duration would further reduce viable counts and probably sterilize experimental bacterial ulcers". To test this Davis et al. (278) continued treatment for 5 days. Animals were sacrificed and corneas excised each day. A 99% reduction in viable counts was seen after the first day, but no significant changes occurred after that, and no eyes were sterile after 5 days of topical tobramycin therapy. The emergence of resistant strains was discounted, as a very small inoculum had been used (20 viable cells). Other workers have also found that the clinical course of the infection does not correlate with the bacteriological status of the infected cornea (280,288). Corneal swab cultures have been used to support clinical assessments (270). Despite the fact that Smolin et al. (285) were of the opinion that "evaluation of the bacteriological status of the cornea from the clinical course of a disease alone may be erroneous", a clinical method was used to evaluate the effect of Anti-LPS plasma treatment on Pseudomonas keratitis in all the animal models

presented in this project.

Clinical assessment may be either qualitative or quantitative. Qualitative scoring methods assign lesions to various groups, describing, for example, as normal, slight, moderate, or marked, such clinical signs as discharge, ulceration, chemosis (284). These groups may alternatively describe the changes in the lesion intensity, eg., unchanged, improved, marked improvement (274). Quantitative assessment, assigning numerical scores to lesions of a particular type, allows statistical comparison of each eye. For example, Galin et al. (212) used a 3 point scale, defined as follows:

(+) = area of corneal involvement less than 1mm diameter; no exudate; inflammed conjunctiva; (described as "minimal")

(2+)= extensive but not complete corneal involvement; exudate; conjunctival inflammation ("moderate")

(3+)= extensive corneal involvement with hypopyon or perforation ("severe")

A widely used method was that described by Wiggins (214, 272, 276). This was a 5 point scale, defined

as follows:

0 = no progress; healed without scarring

1 = central infiltration; healed with faint scar

2 = dense central infiltration; healed with dense central scar

3= more than half cornea infiltrated; healed with dense scar

4 = whole cornea infiltrated; healed with complete vascularized scar

Appreciable changes in the pathology of the infection can occur without the score, as defined above, changing. Gerke et al. (288) expanded this to a 10 point scale, which could record more subtle changes in the corneal lesion. Smolin et al. (267,270) have developed a system which reflects both the size and density of the corneal lesion:

0 = no effect

1+ = up to 33% of the cornea affected

2+ = 34-66% involvement

3+ = 67-100% affected

The score was then adjusted for the density of the lesion: if the opacity was nebulous they added nothing, if macular they added 1 point, if leucomatous 2 points. This resulted in a scale of 0 to 5+. A high degree of correlation was shown

between scores determined by individual observers. The grading system used in the rabbit trials was modified from those described above.

The corneal reaction in guinea pigs has been described by Davis et al. (277). They developed a scoring system which reflected the status of the conjunctiva, corneal epithelium, stroma, corneal vessels, and the anterior chamber. Certain features described as pathological have subsequently been shown to be present in normal healthy guinea pig eyes (287). In the guinea pig trial the results were expressed qualitatively. Eyes were described as either "improved" or "not improved".

4.2 Materials and Methods

4.2.1. Trial 1 : rabbits

Two different inoculation methods were used in this trial, i.e., needle scarification, and contaminated suture techniques.

Group A : needle scarification

In this trial 19 rabbits were infected by the method described by Furgiuele (274). Each eye was pronounced healthy and normal before use. They were then anaesthetised locally with 2 drops of oxybuprocaine 0,4%. The cornea was traumatized by scratching the central area with a sterile 20-gauge needle, in a cross-hatch pattern. The epithelium was removed from the centre of the cross-hatch, in a 5X5mm area. Care was taken so as not to penetrate the anterior chamber. The eye was then inoculated with 2-4 drops of a 24-hour nutrient broth culture of Pseudomonas aeruginosa, containing 600-900 million organisms/ml. The inoculum was allowed to remain in contact with the cornea and inferior fornix for 60 seconds. If no infection

developed over the next 24 hours, the procedure was repeated.

A masked, randomized trial was performed. Treated and control eyes were allocated at random. Where possible (i.e., infection established in both eyes), one eye was treated with Anti-LPS plasma, and the contralateral eye received saline. Treatment was initiated after 48 hours, when the infection was clearly established. The same dosage regimens were used as in the ocular tolerance trials, i.e., 40 drops/minute for 5 minutes, 3 times a day.

The eyes were examined daily, and photographed at the commencement of treatment, on day 4 of the treatment, and after 7 days. The lesions were assessed at the slit-lamp biomicroscope, and from photographic records, and were graded by a system adapted from those used by previous workers (214,267). The following parameters were used:

1. area of cornea involved

2. depth of lesion

3. degree of vascularization

These were graded as shown in Table 1.

Table 1 : Method of scoring severity of experimental Pseudomonas keratitis

CLINICAL SCORES

% surface of cornea affected	Grade (a)	density and depth	Grade (b)	Vascular-ization	Grade (c)
0	(a)0	none/nebulous	(b)0	none	(c)0
1-33	(a)1	mild macular	(b)1	mild	(c)1
34-66	(a)2	moderate leuco-		moderate	(c)2
67-100	(a)3	matous	(b)2	severe	(c)3
		severe leuco-matous	(b)3		
		perforation	(b)4		

Morbidity index = (a)+(b)+(c) (maximum = 10)

Thus (a)0 indicated no infection, (a)1 up to 33% corneal involvement, (a)2 from 34 to 66% surface keratitis, and (a)3 67 to 100% affected. If the opacification was nebulous, the score was (b)0, if macular (b)1, moderate leucomatous (b)2, severe leucomatous (b)3. If the cornea perforated, this was scored as (b)4. Finally, if no vascularization was present, it would be scored as (c)0, if mild (c)1, if moderate (c)2, and if severe (c)3.

Therefore, a moderate keratitis, involving 50% of the corneal surface, with mild opacity, and some vascularization, would be scored as (a)2(b)1(c)1. In assessing the overall severity, the numerical sum of these clinical scores was expressed as the morbidity index (MI), in this case 4. A maximum morbidity index of 10 was thus possible.

Group B : contaminated suture

Five rabbits were used in this trial. Hessburg's contaminated suture technique was used to infect the eyes (276).

Virgin silk sutures (8/0) was soaked for 5 minutes in a 24 hour nutrient broth culture of Pseudomonas aeruginosa. The rabbit eye was anaesthetised as before, and then 2 sutures were passed through the corneal stroma. The ends were cut, and the sutures were left unknotted. Tarsorrhaphy was not performed. The sutures were removed after 2 days.

Treatment was initiated after 18 hours. The same regimen was used, and the lesions were evaluated as before.

4.2.2 Trial 2 : rabbits

A total of 15 rabbits was used for this trial, i.e., 6 severe cases from Trial 1, Group A; the 5 rabbits in Trial 1, Group B; and 4 further rabbits infected as in Trial 1, Group B.

The following treatment regimen was used:
Anti-LPS group : Anti-LPS plasma 3 times a day for 7 days, at a dose of 40 drops/minute for 5 minutes. For the next 7 days, the above dosage was continued, except that 2 drops of dexamethasone were instilled after each plasma lavage.
Control group : saline instead of plasma, in the same manner throughout.

The eyes were photographed on days 0,7, and 14, i.e., before treatment, after 7 days of treatment (before the addition of steroids), and on the last day of the treatment schedule. The extent of the corneal morbidity was graded on a 10 point scale, as described before (Table 1).

4.2.3 Trial 3 : guinea pigs

Thirteen guinea pigs were used in this trial. All had normal corneae and conjunctivae before the trial commenced. The eyes were inoculated as follows:

the guinea pigs were anaesthetized with ether, and then the central cornea was scarified with a 20-gauge needle which had been dipped in a nutrient broth culture of Pseudomonas aeruginosa. Two non-penetrating horizontal scratches were made, and the central epithelium was removed.

Treatment was initiated after 12 hours. The same plasma/saline regimen was used as before. The first 2 animals were left untreated to enable the clinical course of the infection to be observed.

The appearance of the eyes was evaluated on day 7. The appearance of the treated eye was compared to that of the contralateral control eye, and to those results obtained in the preliminary experiment. The eyes were described as either "improved", or "not improved".

4.3 Results

4.3.1 Trial 1 : rabbits

Group A: needle scarification: No infection could be established in either eye of 5 rabbits, despite repeated inoculation. In a further 4 rabbits, infection was only established in one eye. Two were used as saline controls, and 2 received plasma. The latter improved so rapidly, that this was not considered to be due to the plasma. They were therefore deleted from the trial. Overall, no infection could be established in 14/38 (36,8%) eyes. In 10 rabbits, one eye was treated with Anti-LPS plasma, and the contralateral eye with saline.

Group B: contaminated suture: A severe infection developed in 10/10 eyes inoculated with contaminated sutures. Five were treated with Anti-LPS, and the 5 contralateral eyes served as saline controls.

The results therefore reflect the outcome in 15

Anti-LPS treated eyes, compared to 17 saline treated controls. Table 2 shows these results.

Table 2 : Outcome of experimental Pseudomonas keratitis (Trial 1)

Treatment	No of eyes treated	Improved(%)	Unchanged(%)	Deteriorated(%)
Saline	17	4 (23,5)	4 (23,5)	9 (52,9)
Anti-LPS	15	13 (86,7)*	1 (6,7)**	1 (6,7)***

* p = 0,0009 (Fisher's exact probability test)
** p = 0,4160 N.D.
*** p = 0,0119

Of the Anti-LPS treated eyes, 13 improved (86,7%; p = 0,0009), 1 (6,7%) remained unchanged, and 1 (6,7%) deteriorated. Four saline eyes (23,5%) improved spontaneously, 4 (23,5%) remained unchanged, and 1 (52,9%) deteriorated. By day 7, 5 of these had already perforated. The unchanged eyes were already in the most severe group, so further deterioration could only have led to perforation.

Table 3 shows the morbidity indices recorded on days 0,4, and 7.

Table 3 : Morbidity indices of corneal pathology (Trial 1)

		CONTROLS				TREATED			
		DAYS			RESULT*	DAYS			RESULT
	RABBIT No.	0	4	7		0	4	7	
GROUP A	1	9	8	9	0	9	9	7	+
	2	9	9	9	0	9	9	7	+
	3	9	9	10	-	4	6	6	-
	4	5	2	2	+	5	4	4	+
	5	3	2	2	+	9	6	4	+
	6	9	9	9	0	4	2	2	+
	7	6	8	9	-	9	8	6	+
	8	6	8	9	-	5	4	3	+
	9	6	8	9	-	6	6	4	+
	10	6	4	3	+	4	2	2	+
	11	6	8	9	-				
	12	3	2	2	+				
GROUP B	13	8	9	10	-	8	7	6	+
	14	8	9	10	-	8	8	6	+
	15	8	9	10	-	8	8	6	+
	16	8	9	10	-	8	7	6	+
	17	8	8	8	0	9	9	9	0

* + = improvement
0 = no change
- = deterioration

Table 4 : Statistical analysis of morbidity indices (Trial 1)

Treatment	Day	Mean (S.D.)	't'	p
Saline	0	6,9 (2,0)		
Anti-LPS	0	7,0 (2,1)	-0,16	> 0,20
Saline	0	6,9 (2,0)		
Saline	7	7,6 (3,1)	-0,85	> 0,20
Anti-LPS	0	7,0 (2,1)		
Anti-LPS	7	5,2 (2,0)	2,44	< 0,05 *
Saline	7	7,6 (3,1)		
Anti-LPS	7	5,2 (2,0)	2,60	< 0,02 *

* = significantly different

Analysis by Students' 't' test showed that (Table 4):

1. the morbidity indices of the Anti-LPS and saline groups were not significantly different before the trial commenced ('t'=0,16 ; p>0,20)
2. by day 7, the saline treated eyes had deteriorated, although not significantly ('t'=0,85 ; p>0,20)
3. by day 7, the Anti-LPS treated eyes had improved significantly ('t'=2,44 ; p<0,05)
4. at the end of the trial (day 7), the Anti-LPS treated eyes were significantly better than the saline treated controls ('t'=2,60 ; p<0,02)

Table 5 shows the complete clinical scores recorded on days 0 and 7.

Table 5 : Clinical evaluation of rabbit eyes before and after treatment (Trial 1)

	RABBIT No	CONTROLS DAYS 0 (a)	(b)	(c)	7 (a)	(b)	(c)		TREATED EYES DAYS 0 (a)	(b)	(c)	7 (a)	(b)	(c)	
GROUP A	1	3	3	3	3	3	3		3	3	3	2	3	2	
	2	3	3	3	3	3	3		3	3	3	2	2	3	
	3	3	3	3	3	4	3		1	2	1	2	2	2	**
	4	2	2	1	1	1	0	*	2	2	1	1	2	1	
	5	1	1	1	1	1	0	*	3	3	3	1	2	1	
	6	3	3	3	3	3	3		1	2	1	1	1	0	
	7	2	2	2	3	3	3		3	3	3	2	3	1	
	8	2	2	2	3	3	3		2	2	1	1	1	1	
	9	2	2	2	3	3	3		2	2	2	2	1	1	
	10	2	2	2	1	1	1	*	1	2	1	1	1	0	
	11	2	2	2	3	3	3								
	12	1	1	1	1	1	0	*							
GROUP B	13	3	2	3	3	4	3		3	2	3	2	2	2	
	14	3	2	3	3	4	3		3	2	3	2	2	2	
	15	3	2	3	3	4	3		3	2	3	2	2	2	
	16	3	2	3	3	4	3		3	2	3	2	2	2	
	17	2	3	3	3	2	3		3	3	3	3	3	3	**

* = control eyes that improved

** = treated eyes that deteriorated or did not improve

(a) = area of keratitis; (b) = depth of lesion ;
(c) = degree of vascularization

Figure 1 shows the eye of rabbit 7 treated with Anti-LPS plasma, at day 0 (A), and at day 7 (B).

Figure 2 shows the contralateral control eye, at the same stages (A and B).

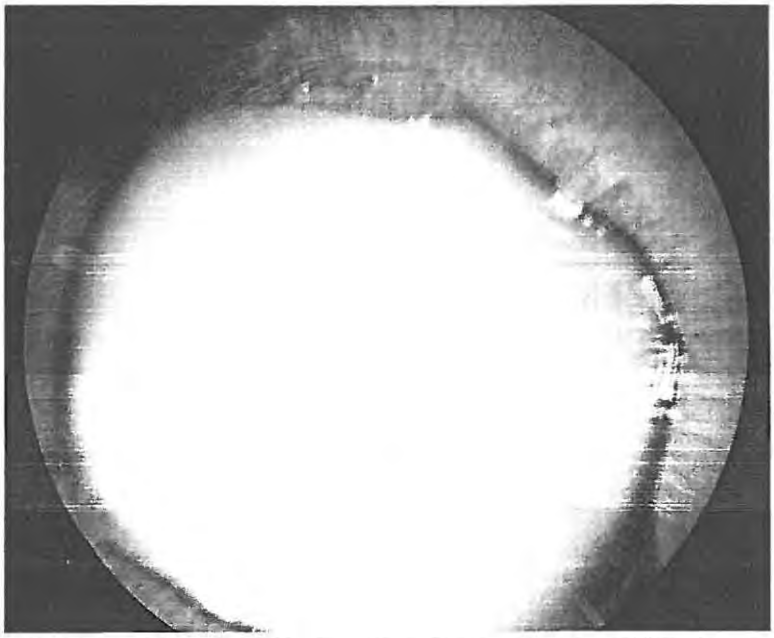

Figure 1A: Rabbit eye infected with Pseudomonas aeruginosa. Note extensive opacification and infection.

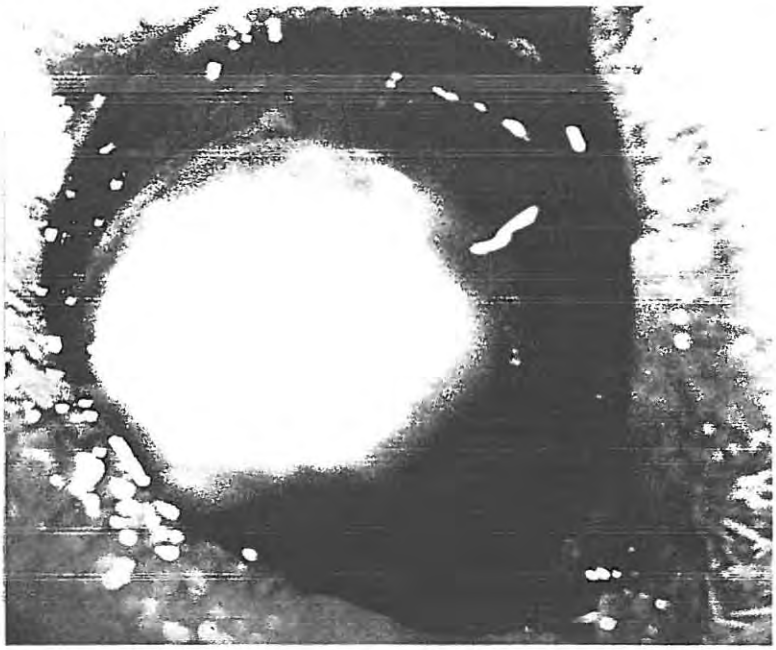

Figure 1B: Same eye after 7 days of Anti-LPS therapy. The extent of keratitis and scarring is much reduced, with control of infection.

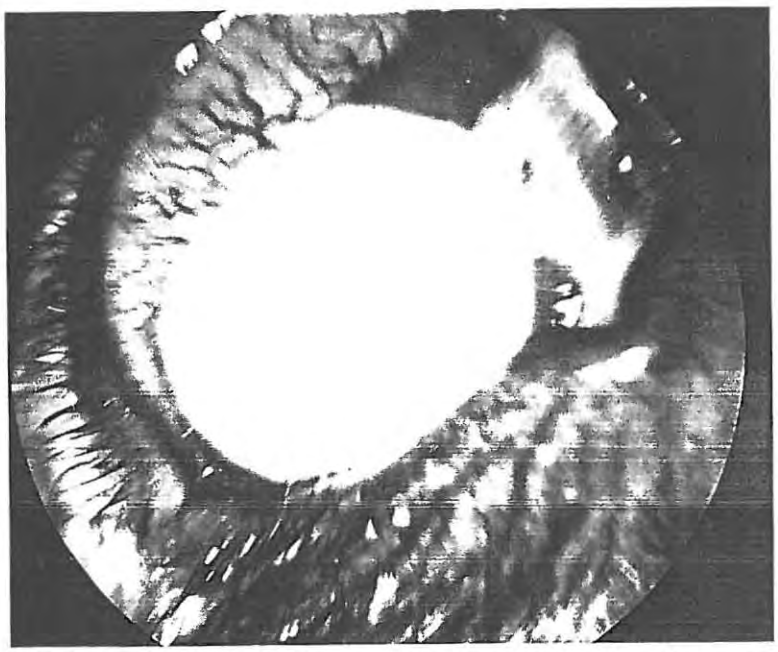

Figure 2A: Contralateral control eye. Similarly infected.

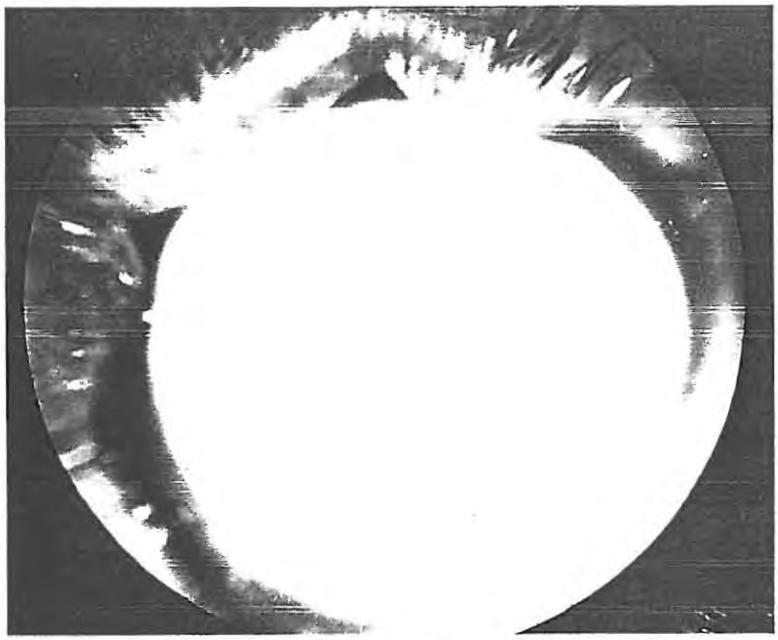

Figure 2B: Same eye after 7 days of saline treatment. The infection is still active and the eye is extremely inflamed.

4.3.2 Trial 2 : rabbits

A severe infection developed in all 30 eyes inoculated by either needle scarification or contaminated sutures.

Table 6 shows the results of the trial, at day 7, and at day 14.

Table 6 : Outcome of experimental Pseudomonas keratitis (Trial 2)

Treatment (Day)	No. of eyes treated	Improved (%)	Unchanged (%)	Deteriorated (%)
Saline (7)	15	2(13,3)	4(26,70	9(60,0)
Anti-LPS(7)	15	12(80,0)*	1(6,7)**	2(13,3)***
Saline/cort (14)	15	1(6,7)	3(20,0)	11(73,3)
Anti-LPS/ cort (14)	15	12(80,0)#	1(6,7)##	2(13,3)###

* p = 0,0007 (Fisher's exact probability test)
** p = 0,3295 N.D.
*** p = 0,029

\# p = 0,0001
p = 0,5977 N.D.
p = 0,0025

After 7 days of Anti-LPS treatment, 12/15 (80,0%) eyes had improved, while 1/15 (6,7%) remained unchanged, and 2/15 (13,3%) had deteriorated. Of the 15 contralateral control eyes, 2 (13,3%) had improved, 4 (26,7%) had remained unchanged, and 9 (60,0%) had deteriorated. The improvement in the two groups was significantly different ($p = 0,0007$). After the addidition of corticosteroids to the treatment regimen, after 14 days, the number of improved Anti-LPS eyes (12/15 ; 80,0%) was still significantly greater than those of the controls (1/15 ; 6,7% ; $p = 0,0001$). Of the controls, 3 (20,0%) remained unchanged, and 11 (73,3%) had deteriorated. One (6,7 %) Anti-LPS/steroid treated eye remained unchanged, while 2 (13,3%) had deteriorated.

However, the steroid had resulted in a significant improvement in the morbidity indices of the Anti-LPS group. These are shown in Table 7.

Table 7 : Morbidity indices of corneal pathology*

(Trial 2)

Rabbit No.	CONTROL Days 0	7	14	Result #	TREATED Days 1	7	14	Result #
1	3	2	2	+	9	4	3	+
2	9	9	9	0	9	6	3	+
3	9	9	9	0	9	7	3	+
4	6	9	9	-	6	4	2	+
5	9	9	10	-	9	7	3	+
6	9	10	10	-	4	6	5	-
7	8	10	10	-	8	6	4	+
8	8	10	10	-	8	6	5	+
9	8	10	10	-	8	6	5	+
10	8	10	10	-	8	6	5	+
11	8	8	8	0	9	9	9	0
12	5	9	8	-	6	7	7	-
13	5	8	10	-	5	3	2	+
14	9	9	10	-	9	7	6	+
15	7	6	10	-	7	5	3	+

* summation of scores (a)+(b)+(c)
\# + = improvement
0 = no change
- = deterioration

Table 8 shows the complete clinical scores.

Table 8 : Clinical evaluation of Pseudomonas keratitis (Trial 2)

No.	CONTROL EYES									TREATED EYES								
	Day 0			Day 7			Day 14			Day 0			Day 7			Day 14		
	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)	(a)	(b)	(c)
1	1	1	1	1	1	0	1	1	0*	3	3	3	1	2	1	1	2	0
2	3	3	3	3	3	3	3	3	3	3	3	3	2	3	1	1	2	0
3	3	3	3	3	3	3	3	3	3	3	3	3	2	3	1	1	2	0
4	2	2	2	3	3	3	3	3	3	2	2	2	2	1	1	1	1	0
5	3	3	3	3	3	3	3	4	3	3	3	3	2	2	2	1	1	1
6	3	3	3	3	4	3	3	4	3	1	2	1	2	2	2	2	2	1#
7	3	2	3	3	4	3	3	4	3	3	2	3	2	2	2	2	2	0
8	3	2	3	3	4	3	3	4	3	3	2	3	2	2	2	2	2	1
9	3	2	3	3	4	3	3	4	3	3	2	3	2	2	2	2	2	1
10	3	2	3	3	4	3	3	4	3	3	2	3	2	2	2	2	2	1
11	3	2	3	3	2	3	3	2	3	3	3	3	3	3	3	3	3	3#
12	2	3	0	3	3	3	3	3	2	2	3	1	3	3	1	2	2	3#
13	2	2	1	3	3	2	3	4	3	2	2	1	1	1	1	1	1	0
14	3	3	3	3	3	3	3	4	3	3	3	3	3	2	2	3	2	1
15	3	2	2	2	2	2	3	4	3	3	2	2	1	2	2	1	2	0

* = control eye that improved

\# = treated eye that deteriorated or did not improve

(a) = area of keratitis; (b) = depth of lesion; (c) = degree of vascularization

The morbidity indices were analysed by Students' 't' test. The results are shown in Table 9.

Table 9 : Statistical analysis of morbidity indices (MI) (Trial 2)

Group	Mean MI	(S.D.)	vs Group	Mean MI	(S.D.)	't'	p	
X(0)	7,4	(1,8)	Y(0)	7,6	(1,6)	-0,31	> 0,20	
X(0)	7,4	(1,8)	X(7)	8,5	(2,1)	-1,57	< 0,20	
X(7)	8,5	(2,1)	X(14)	9,0	(2,1)	-0,61	> 0,20	
X(0)	7,4	(1,8)	X(14)	9,0	(2,1)	-2,24	< 0,05	*
Y(0)	7,6	(1,6)	Y(7)	5,9	(1,5)	2,92	< 0,01	*
X(7)	8,5	(2,1)	Y(7)	5,9	(1,5)	3,91	< 0,01	*
Y(7)	5,9	(1,5)	Y(14)	4,3	(2,0)	2,53	< 0,02	*
Y(0)	7,6	(1,6)	Y(14)	4,3	(2,0)	4,96	< 0,01	*
X(14)	9,0	(2,1)	Y(14)	4,3	(2,0)	6,35	< 0,01	*

* = significantly different

X(0) = control eyes at day 0 Y(0) = Anti-LPS eyes at day 0
X(7) = control eyes at day 7 Y(7) = Anti-LPS eyes at day 7
X(14) = control eyes at day 14 Y(14) = Anti-LPS eyes at day 14

Statistical analysis by Students' 't' test revealed the following:

1. the mean morbidity indices (MI) of the control and Anti-LPS groups were not significantly different before the trial commenced ('t'=0,31 ; p>0,20)
2. control eyes deteriorated significantly by day 14, compared to day 0 ('t'=2,24 ; p<0,05)
3. Anti-LPS treated eyes improved significantly by day 7 ('t'=2,92 ; p<0,01)
4. at day 7, treated and control eyes were significantly different ('t'=3,91 ; p<0,01)

5. the addition of corticosteroids caused a significant improvement in the Anti-LPS treated eyes ('t'=2,53 ; $p<0,02$), whereas control eyes continued to deteriorate

6. the overall improvement in the anti-LPS/steroid treated eyes was significant ('t'=4,96 ; $p<0,01$)

7. the ultimate appearance of the treated eyes was significantly better than that of the contralateral control eyes ('t'=6,35 ; $p<0,01$)

Figure 3 shows the eye of rabbit 13, treated with Anti-LPS and steroid drops, at day 0 (A), 7 (B), and 14 (C). Figure 4 shows the contralateral control eye at the same stages (A, B, and C).

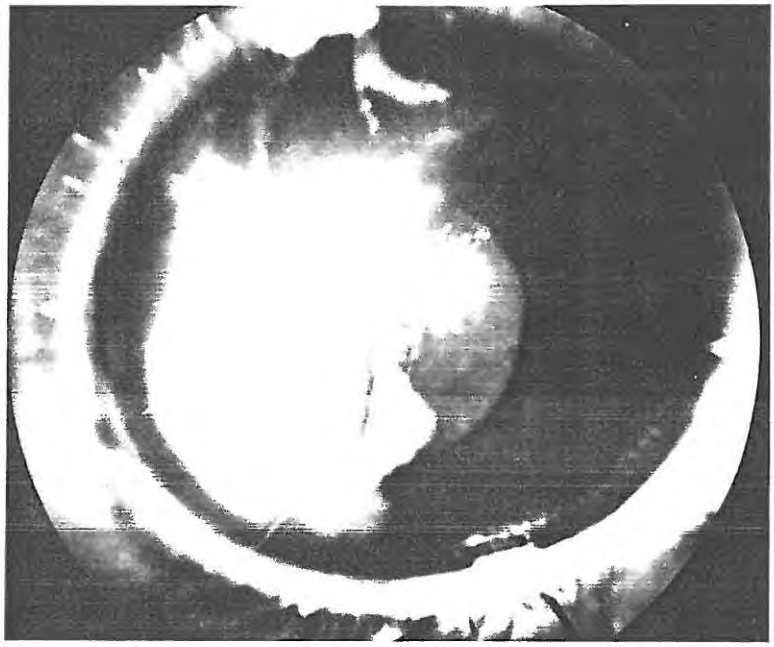

Figure 3A: Rabbit eye heavily infected with Pseudomonas aeruginosa. Note extensive infiltration and opacity.

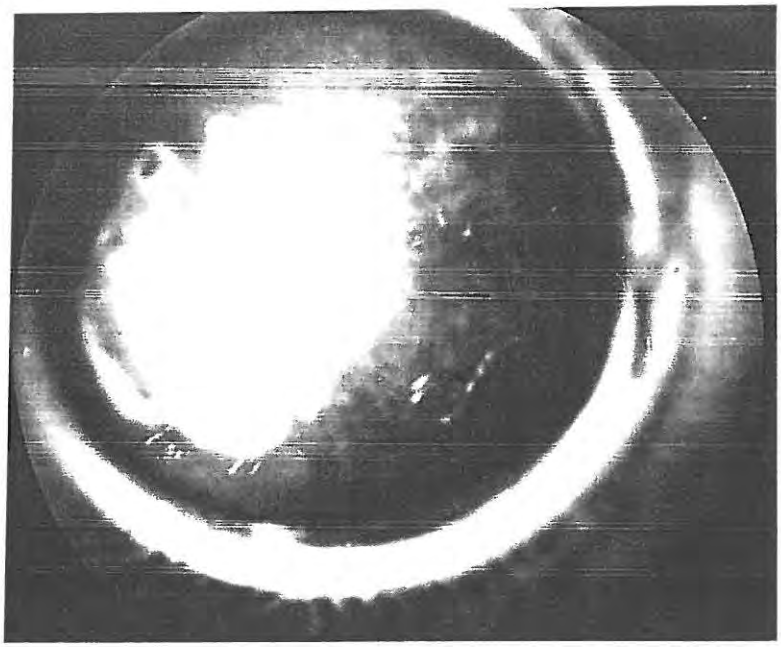

Figure 3B: Same eye after 7 days of Anti-LPS therapy. The infection is under control and the extent of keratitis is much reduced.

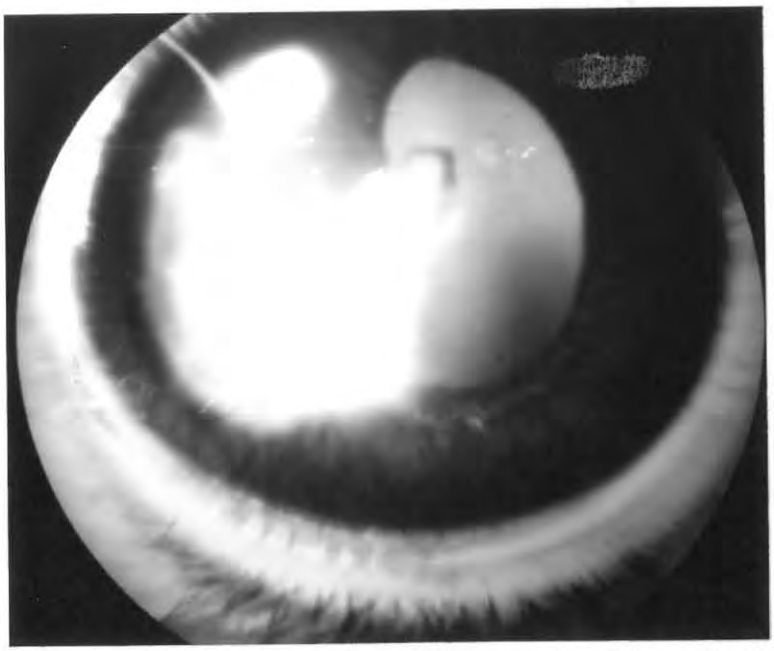

Figure 3C: Same eye after a further 7 days of Anti-LPS/steroid therapy. Note dramatic improvement in the extent of infection and inflammation.

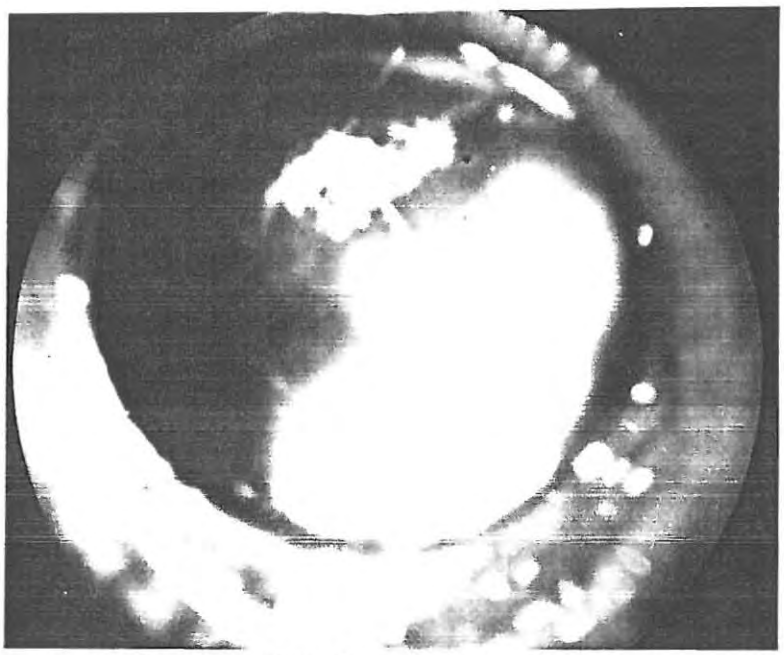

Figure 4A: Contralateral control eye. Similarly infected.

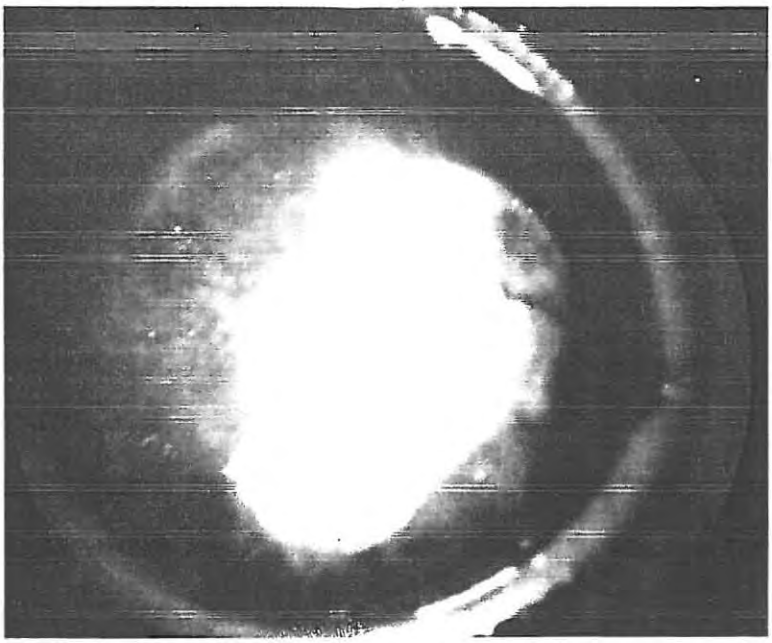

Figure 4B: Same eye after 7 days of saline treatment. The infection has worsened slightly.

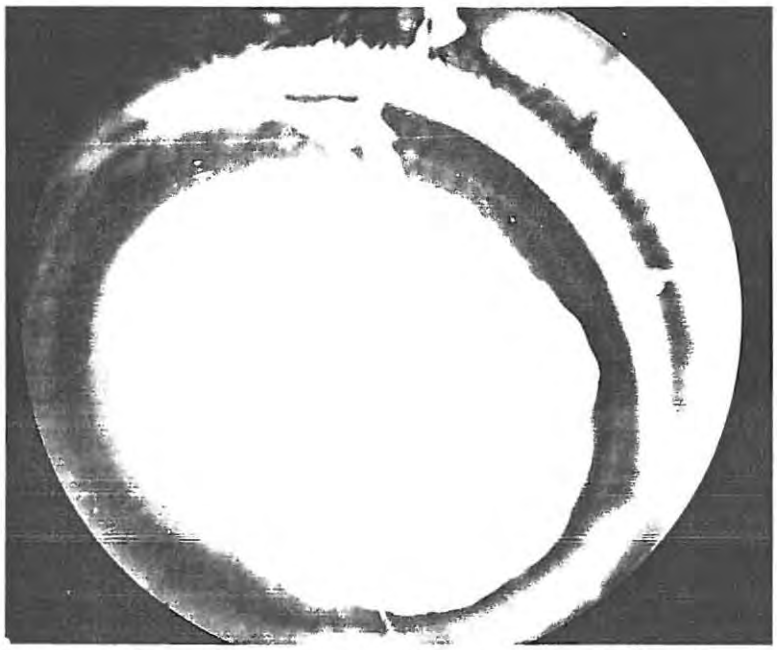

Figure 4C: Same eye after a further 7 days of saline/steroid treatment. The eye is necrotic, and has perforated at the limbus.

4.3.3 Trial 3 : guinea pigs

Guinea pigs behaved differently from rabbits, in that corneal infections of consistent intensity were induced in all 15 animals inoculated by needle scarification. The infections obtained were similar in the left and right eyes of each animal. The course of the infection in the 2 left untreated was similar to that described by Davis et al. (277). The infection progressed until day 7, being worst at day 5. At this stage the corneal infection was still suppurative, and presented as a totally infiltrated, opaque, vascularized eye. Thereafter the infection subsided, and the eye was usually "quiet" by day 14, with a dense, leukomatous scar. The appearance of the eyes was therefore evaluated on day 7 of treatment. Treated eyes were described as being either "improved" or "not improved". Eyes described as "improved" had no discharge at day 7, compared to controls, and healed with only nebulous scarring, compared to the dense scars on the control eyes.

Two guinea pigs were deleted from the trial as a

low titre plasma had been accidentally used to treat them, and the results were not comparable. A total of 11 animals were included in the trial. The results are shown in Table 10.

Table 10: Treatment of Pseudomonas keratitis in guinea pigs (Trial 3)

Treatment	No. of eyes treated	Improved (%)	Not improved (%)
Saline	11	1 (9,1)	10 (90,9)
Anti-LPS	11	7 (63,6)*	4 (36,4)

* p = 0,0237 (Fisher's exact probability test)

While none of the Anti-LPS treated eyes cleared completely, 7/11 (63,6%) improved significantly compared to the saline treated controls. Of the control eyes, only 1/11 (9,1%) improved in the same manner. This incidence was significantly different ($p = 0,0237$). In one case the infection in the control eye apparently spread into the systemic circulation, as evidenced by gross facial oedema on the right side, and the subsequent death of the animal. However, the plasma treated eye had already improved, and a significant difference between the left and right eyes was noticeable. Figure 5 shows the ultimate appearance of the eyes of guinea pig 4, treated with Anti-LPS (A), and saline (B).

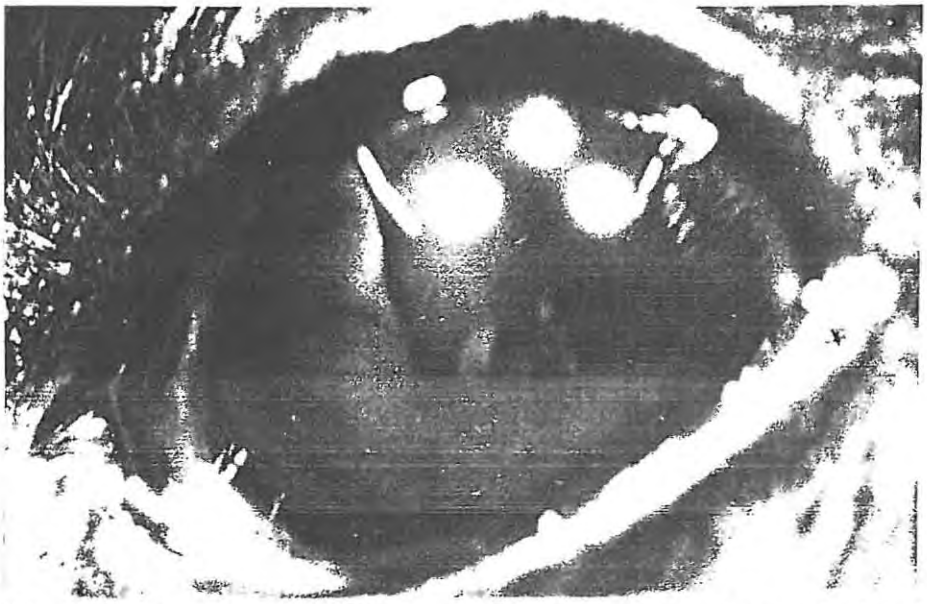

Figure 5A: Anti-LPS treated eye, showing minimal scarring.

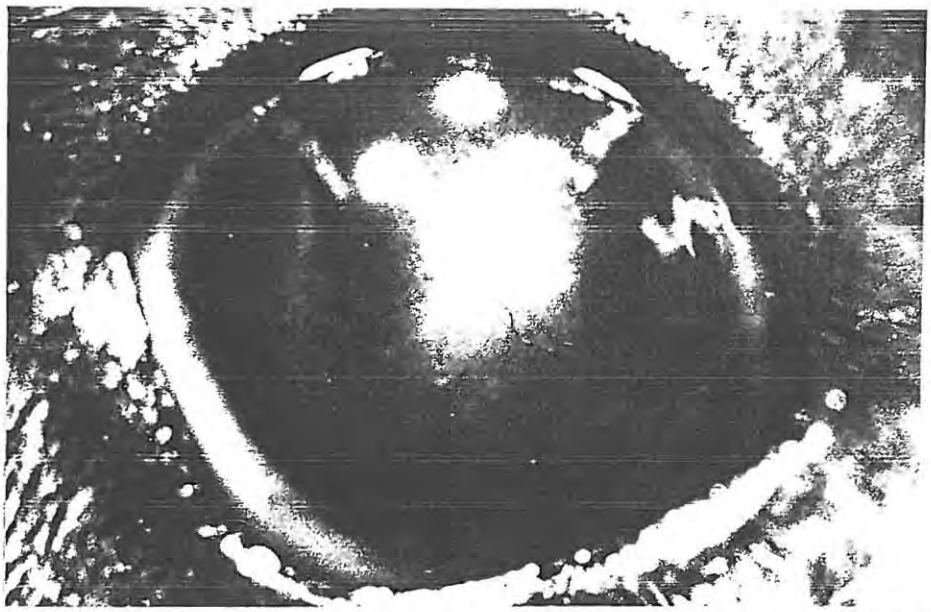

Figure 5B: Saline control eye, with large vascular scar.

4.4 Discussion

4.4.1 The experimental model

A common problem with any experimental model is that of reproducibility. An early report on the rabbit model of Pseudomonas keratitis concluded that this animal was unsuitable for testing antimicrobials because of the difficulty of obtaining a 100% 'take' of infection following needle scarification (289). Nevertheless, rabbits has continued to be used by many researchers, up to the present day (290).

The method first used, in Trial 1, was that described by Furgiuele (275). No infection could be established in 14/38 (36,8%) eyes so inoculated. This was not significantly different from the figures reported by Furgiuele, who could establish no infection in 14/48 (29,2%) rabbit eyes. In a subsequent report he again referred to the fact that this method resulted in infections of varied severity (284). Various reasons have been proposed, the most likely being prior exposure of the animal to Pseudomonas, enhancing natural

immunity. A genetic basis for susceptibilty has been shown in mice, but not in rabbits (291,292). Studies in mice have shown that, provided the initial injury is sufficient, the absolute extent and means of inflicting the wound do not have a significant influence on the outcome of the infection (288). Extreme variability in the response to inoculation by needle scarification was found in this study. Gerke also found that inoculum size did not correlate with the severity of the subsequent infection. It was therefore decided not to standardize the inoculum size exactly, and 24 hour cultures containing 600-900 million organisms/ml were used, as prescribed by Furgiuele (274).

The severity of the infection does reflect, in part, the virulence of the particular strain responsible. The strain used in this project was obtained from a stock culture collection, and was of unknown virulence. Use of an extremely virulent strain from a nosocomial burn wound infection has been reported to cause an infection in all rabbits inoculated by needle scarification (272). The

effect of strain virulence has also been noted in studies which used the Cignetti inoculation method (abrasion with a toothed chalazion curette). One group used sub-conjunctival injections of corticosteroids to increase the susceptibility of the experimental animals to less virulent strains (285). Variation in response to the Cignetti method has been reported as well (270). For this reason, the Cignetti method was not used in this project. Moreover, the needles used had the additional advantage of being sterile, disposable, and cheap. The needle scarification method also more closely resembles the clinical situation (290). I attempted a trial using baboons, but no infection could be established by the needle scarification method. The problems of handling such animals precluded further experimentation.

An equally important cause of infection failure in the rabbit is the washing action of the tears, lids, and nictitating membrane, which prevents bacterial adherence and invasion (275). This is negated by methods which introduce the inoculum directly into the corneal stroma. In an effort to

increase the success rate, in terms of establishing a reproducible infection in both eyes of all rabbits inoculated, the method described by Hessburg was used, as adapted by Galin (212,275). Hessburg used a suture, soaked in the inoculum and 'dragged' through the corneal stroma, to introduce bacteria directly into the cornea. Since the strain used in this project was of apparently low virulence, it was decided to leave the suture in the cornea, as did Galin et al. The use of this technique resulted in a severe, fulminant infection in all 18 eyes so inoculated (Trials 1 and 2). Guinea pigs are known to be more susceptible to Pseudomonas keratitis than rabbits, and it was decided to repeat Trial 1 in these animals (Trial 3).

Pseudomonas infections in rabbits are usually self-limiting, unless very severe (214). However, evaluation of the result after 7 days gave an accurate reflection of the status of the infection. More severe infections, such as those caused by the contaminated sutures, more closely mimicked the human clinical situation, in that most of the

control eyes perforated within 14 days, i.e.,a progressive infection was obtained. Gerke concluded that, despite the differences between the human situation and that seen in experimental animals, this did not depreciate the value of the animal infection (288).

An important aspect of any experimental model is the use of adequate controls. Where possible, the contralateral eye served as the control. However, variations in pathology may occur even between contralateral eyes. Overall, at the beginning of the trial, there was no significant difference between the control and test groups, in terms of mean morbidity indices. Several trials have in fact used different rabbits for control purposes, treating both left and right eyes in the same manner (180,267,270,276,279,283,285).

Throughout this study animals of mixed, non-inbred strains were used. Although Davis et al (277,278) have suggested that a specific inbred strain of laboratory animal should be used in order to standardize the model as much as possible, this was not considered necessary. The spectrum of severity

obtained reflects the situation as seen in normal clinical practice (288).

The characteristics of Anti-LPS plasma restricted its use in the therapy of keratitis to the topical route. The antibody concentration was relatively low, 1,5mg/ml, and thus large dose volumes were considered necessary. Being an equine product, IV use in other animals was contraindicated. The antibody molecule is large, so penetration was expected to be limited to the infected area. The variation in response among different animals, treated in the same manner, prevented the determination of an accurate dose-response relationship. An empirically determined dose was first shown to be tolerated by healthy rabbits and baboons (vide 3.3), and this was then used therapeutically as well.

Topical antibiotics used in the treatment of Pseudomonas keratitis are usually administered on an hourly basis, or more often. This has been shown in animal trials to be the most effective method of using gentamicin, tobramycin, and carbenicillin (278,279). These trials were

evaluated in terms of the numbers of surviving Pseudomona cells in the cornea after a 24 hour treatment period. However, in an earlier trial, clinical evaluation over an extended period (8 days) had shown topical administration on a 6-8 hourly basis to be effective, compared to untreated controls (214). Nevertheless, more frequent administration of Anti-LPS plasma would be expected to increase its efficacy against Pseudomonas keratitis.

The dosage regimen used was more a lavage than true topical administration, which implies the instillation of a few drops into the conjunctival sac. Wiggins (214) used 5 minute corneal baths with saline solutions of polymixin B, but could show no advantage over topical administration. In contrast, Hessburg et al (275,276) has shown lavages to be effective in both animals and man. Saline washes were shown to be of benefit to the control eyes in their series, presumably because of the removal of purulent, necrotic material, exoenzymes, and exotoxins. For this reason, the control eyes in this project received the same dose

of sterile, normal saline to counteract the purely physical effects of the plasma lavage, and enable accurate conclusions to be drawn regarding the specific action of Anti-LPS. Many trials of antibiotic therapy have used untreated eyes as controls, although saline is more commonly used (214,274,280).

Studies of Pseudomonas adherence to injured corneal cells have shown that this process is time dependent (19). Washing the cornea within 4 hours of inoculation prevents penetration of the stroma. Topical administration of polymixin B has been shown to prevent progression of the infection if initiated 6 hours after inoculation by the abrasion method (214). If however, treatment was delayed for 18 or 24 hours, the treated eyes healed, but with severe scarring. On the other hand, untreated control eyes perforated. When the Hessburg suture technique was used, acceptable results were obtained if treatment was initiated after 6-8 hours (212). The majority of patients present for treatment approximately 16 hours after the infection has established itself, because at this

stage abcess formation is usually evident (293). Most trials involving topical administration therefore initiate such treatment 24 hours after inoculation (274,278,279). Growth studies have shown that the Pseudomonas organisms in the corneal tissue reach a steady state phase at this time (279). In Trial 1, Group A (needle sacrification), if no frank keratitis was evident after 24 hours, the procedure was repeated. Treatment was thus initiated after 48 hours, when the infection was clearly established. In Group B (contaminated sutures) a full clinical picture was seen within 12 hours. Treatment in this trial was therefore initiated after 18 hours. This method is so severe that Hessburg et al. (276) started lavages 2 hours after inoculation. Control eyes given saline lavages usually progressed to perforation within 7 days. Guinea pigs (Trial 3) were more susceptible to needle scarification than rabbits, and an infection was established in all eyes so inoculated. Treatment was initiated after 12 hours. Clinically, it has been conclusively proven that early intervention improves the chances of success in cases of Pseudomonas keratitis

(146,148). However, a clearly established infection was needed before a valid experiment could be performed, and treatment was thus delayed. Individual cases also demand individualized management, but this is not possible within an experimental series.

The experimental models used in all 3 trials were comparable to those previously reported, and gave an accurate measure of the effect of topical Anti-LPS plasma therapy on Pseudomonas keratitis in vivo. No significant difference between rabbits and primates has been found in terms of reaction to ocular injuries, despite the differences in lid anatomy and blinking physiology (294).

4.4.2 The effect of Anti-LPS plasma

The following is a description of the clinical course of a typical Pseudomonas corneal ulcer in a rabbit, treated with Anti-LPS (see Figure 1):

1. Day 0 (48 hours post-inoculation) : the eye presents as a large ulcer, extending over almost the entire corneal surface. The cornea is densely opaque with loss of the red reflex, and there is an

extensive purulent discharge. The epithelium is largely destroyed, leaving a soft necrotic surface. There is limbal pannus and the conjunctiva are extremely inflamed. The lids are swollen and inflamed.

2. Day 4 of treatment : there is very little discharge. The ulcer is smaller with well defined margins. The cornea is still densely opaque, but is clearing slightly. The conjunctiva are still very inflamed (hypopyon is visible in very severe cases).

3. Day 7 of treatment : no discharge or active infection is seen. There is a leucomatous scar covering less than 66% of the corneal surface, with minimal vascularization. The epithelium has reformed. The conjunctiva are still inflamed, but less so than at days 0 or 4.

The improvement noted in the corneal infections treated with Anti-LPS involved all 3 parameters measured, area of keratitis, depth of lesion, and degree of vascularization. Statistical analysis of the mean morbidity indices (MI) showed that a significant improvement in the Anti-LPS treated eyes occurred by day 7 ('t' = 2,44 ; $p < 0,05$). At

the end of the treatment period there was a significant difference between the Anti-LPS treated eyes and the saline controls ('t' = 2,60 ; $p < 0,02$). In milder cases the plasma was effective, with reversal of the lesion (morbidity index of 3 or less). However, most of the eyes in Trial 1 were severely infected (morbidity index of 6 or more). In these cases the plasma was able to bring the infection under control, and the final result was a firm scar. Control eyes progressed as described by Van Horn et al. (274). The severity of the infections is shown by the fact that 5/17 control eyes perforated by day 7. Of these, 4 were in Group B (contaminated sutures).

In Trial 3, a preliminary experiment was conducted in which 2 guinea pigs were left untreated to enable the clinical course in this model to be observed. The infections produced followed the same course as that described by Davis and Chandler (277), and by Van Horn et al. (295). The typical picture 24 hours after inoculation was that of a severely inflamed eye with total corneal opacity, producing copious amounts of purulent discharge. Lesion severity was maximal at 5-7 days.

Thereafter spontaneous improvement occurred, leaving a dense, vascularized central scar. Evaluation at day 7 was therefore used as the endpoint.

Anti-LPS therapy was seen to accelerate this process, with a quiet eye obtained by day 7. The scars formed were firm, usually macular, and not as severely vascularized. Control eyes still showed total opacity with severe inflammation. Many controls formed descemetoceles, although none perforated.

As mentioned before (vide 4.4.1), the purely physical action of a lavage has been shown to exert a beneficial influence on the outcome of Pseudomonas keratitis. In this series, although some saline treated eyes did improve, the mean morbidity index for the rabbit control group (Trial 1) appeared numerically worse at the end of the trial period, but this was not statistically significantly ('t' = 0,85 ; p $\frac{1}{2}$ 0,20). What was important was that there was no significant improvement in this group. The improvement noted in the Anti-LPS treated groups could therefore only

be attributed to the specific properties of the plasma.

A dual mechanism of action is presumably involved (297):

1. inactivation and opsonization of released LPS
2. killing of viable bacterial cells.

Inactivation is accomplished by binding of the antibodies close to the toxic site of the "free" LPS. Many antigen-antibody complexes has been shown to be eliminated more efficiently by the RES (296). The exact role of LPS in the pathogenesis of Pseudomonas keratitis has not been fully elucidated (vide 1.2.4.2). LPS has been implicated in the disease process by a number of researchers (71,97,107,298). Inactivation of the LPS released by the infecting organism would therefore be of benefit to the infected eye, since LPS may be partially responsible for the influx of PMN's, which produce cornea-damaging enzymes (299, 300).

Anti-LPS antibodies also bind to LPS on the surface of Gram negative bacteria. This binding activates

complement, which damages the bacterial cell wall by enzymatic digestion, resulting, ultimately, in cell lysis. This type of bactericidal action has been demonstrated for E.coli (301-304).

The specific bactericidal action of Anti-LPS antibodies has been demonstrated by Gaffin et al. (297), in studies using Klebsiella pneumoniae. Pseudomonas aeruginosa is usually resistant to the bactericidal effects of fresh pooled serum (77). This was found to be the case in 91% of Pseudomona blood isolates, compared with only 14% of E.coli stool isolates. Bjornson and Michael (74) showed that natural IgG antibodies, properdin, and C3 proactivator were normally required for the opsonophagocytosis of Pseudomonas by human PMN's. The addition of immune IgG antibodies, prepared by immunization with Fisher's polyvalent LPS vaccine, eliminated the need for properdin and C3PA. Bishop et al. (242) found that antibodies to the slime glycoprotein could opsonize Pseudomonas cells and thus enhance their phagocytosis by PMN's. This system was most effective in the presence of complement. Interestingly, antibiotic resistant strains of Pseudomonas have been found to be more susceptible to PMN phagocytosis (305).

It would thus seem that this dual action of Anti-LPS (antitoxic and bactericidal), combined with the physical action of the lavage-type method of administration, is effective against Pseudomonas keratitis, in the experimental models used in this project.

4.4.3 The effect of corticosteroids

The use of corticosteroids to reduce inflammation and to aid in healing is accepted in many areas of medicine. Their use in ocular infections has been contraindicated by some authors, because of reported cases of relapse of the infection (306). Prolonged use of steroid drops may result in local immunosuppression, and thus predispose to the occurrence of various bacterial and fungal corneal infections (10,14,80). Systemic steroid use has been blamed for a poor result in a case of Pseudomonas infected exposure keratitis in a semicomatose patient (66). However, other workers have reported good results when steroids have been incorporated in the treatment regimen (154,307). In his comprehensive review on corneal and scleral

infection, Laibson concluded that steroids "should not be recommended without cautioning the ophthalmologist about the dangers of this drug's use and the always present threat of a recurrent Pseudomonas ulceration after apparent healing" (1). He did however acknowledge that corticosteroids could, if used properly, be of benefit by allowing the eye to "quiet" and saving the eye from perforation.

Animal studies have also revealed conflicting results. Beneficial effects were noted in experimentally-induced Staphylococcus aureus endophthalmitis (308,309). Prednisolone was shown to have no effect on the efficacy of tobramycin and carbenicillin therapy for Pseudomonas keratitis in guinea pigs (310). However, this was based purely on the number of viable organisms remaining in the cornea after 24 hours' treatment. The use of triamcinolone acetonide was shown to slow the healing of corneal lesions in a rabbit trial using a Pseudomonas resistant strain of Pseudomonas (311). Nevertheless, the end result was the same, in terms of ultimate scarring and inflammation.

Many of the adverse effects of corticosteroids are only evident after high dose, prolonged administration. Quantitative mesurements of the effect of topical dexamethasone treatment on corneal wound healing (in terms of tensile strength) showed a significant effect only after doses of 0,1% drops every hour, for 12 hours a day, over a period of 11 days (312). Similarly, only high doses of hydrocortisone were able to suppress complement (313) and PMN activity (314). However, only 1 subconjunctival injection of hydrocortisone was shown to reduce the number of PMN's in non-infected trephine wounds in rabbit corneas, for up to 6 hours (315).

In Trial 2, a significant improvement was noted when corticosteroids were added to Pseudomonas infected rabbit eyes, treated with Anti-LPS plasma ('t'= 2,53 ; $p < 0,02$). As in Trial 1, plasma treatment resulted in a significant improvement in overall morbidity by day 7 ('t'= 2,92 ; $p < 0,01$). Closer analysis of the clinical scores (Table 8) shows that this improvement was largely in the area of the lesion (a), and the degree of vascularization (c). Both of these had declined

significantly by day 7 ('t'= 2,87 ; p < 0,01; and 't'= 2,60 ; p < 0,02 respectively). The depth of the lesion did not improve significantly ('t'= 1,57 ; p < 0,20), as determined by the grading system used. However, scar tissue was forming, and the epithelium had reformed in most cases. These changes could not be reflected in the clinical scores, as defined. The addition of steroids to the regimen led to an improvement in all 3 parameters measured. Only the reduction in inflammation was statistically significant, as graded by the system described earlier (vide 4.2.1) ('t'= 2,93 ; p < 0,01). However, the overall improvement compared to day 0 was significant for all 3 parameters, area of keratitis ('t'= 4,07 ; p < 0,01), depth of lesion ('t'= 3,18 ; p < 0,01), and degree of vascularization ('t'= 4,92 ; p < 0,01). In contrast, saline treated control eyes deteriorated significantly by day 7 ('t'= 2,92 ; p < 0,01), and continued to deteriorate until day 14 ('t'= 2,53 ; p < 0,02). Overall the mean MI of the control eyes was significantly worse at day 14, compared to day 0 ('t'= 4,96 ; p < 0,01), and to the Anti-LPS treated eyes at day 14 ('t'= 6,35 ; p < 0,01) (Table 9).

The beneficial effects of corticosteroid therapy can be explained in terms of recent proposals concerning the mechanisms of corneal damage. Studies in mice using heat-killed inocula have shown that corneal damage depends not only on an influx of viable cells, but also on host PMN influx (300). When pretreated with cyclophosphamide the mice could not mount a PMN response, and no changes in corneal morphology were noted. The changes seen in untreated mice were due to host- derived factors, since no normal Pseudomonas exoproducts were being produced (vide 1.2.4). Kessler et al. (299) were able to show a correlation between the levels of PMN-derived collagenase and proteoglycan-degrading proteolytic enzymes, and the extent of corneal damage caused by inoculation with heat-inactivated Pseudomonas aeruginosa. The authors were of the opinion that the PMN response was initiated by either Pseudomonas LPS or slime polysaccharides, both being heat stable. The effects of steroids on PMN infiltration (315) would therefore prevent corneal damage by this mechanism.

However, a PMN response is crucial in the early stages of therapy with Anti-LPS plasma, as shown

above (vide 4.4.2). For this reason, dexamethasone was introduced only after 7 days, by which time the infection was under control. At this stage the viable counts in the cornea could be expected to be very low, but host enzyme mediated inflammatory reactions would still be significant, in terms of tissue damage and ultimate scar formation (273). Plasma was therefore still administered for a further 7 days in order to prevent a recurrence of the infection, due to multiplication of a few residual organisms (273,278,306). In this way dramatic improvement was obtained, and partial healing occurred. No Anti-LPS treated eyes perforated, and most formed firm scars with minimal vascularization. No relapse was seen in the Anti-LPS group. Saline treated control eyes continued to deteriorate, and 11/15 had perforated by day 14.

It appears that the use of corticosteroids, once the infection is under control, and with appropriate Anti-LPS bactericidal cover, has a beneficial effect on the outcome of Pseudomonas keratitis, in this experimental model. Human clinical trials are needed to develop this therapeutic regimen as a possible addition to the ophthalmologist's armamentarium.

CHAPTER 5

5. IN VITRO STUDIES OF ANTI-LPS PLASMA ACTIVITY

5.1 Introduction

Various methods have been developed to measure the activity of antimicrobial agents in vitro. The classical method is the agar zone diffusion technique, which has been comprehensively reviewed by Linton (316). A zone of inhibition of bacterial growth is seen around the well cut into the agar surface which contains the antbacterial agent. Where no antibacterial action occurs, bacterial growth extends to the edge of the well. The size of the zone of inhibition is direct reflection of the activity of the agent against the particular strain of bacteria.

An important factor in determining the usefulness of any antibacterial agent against a bacterial species, is the minimum inhibitory concentration of the agent for that bacterium. The actual concentration achieved in vivo by any route of

administration must be able to exceed the MIC for the agent to be effective in that mode. A commonly used method of determining the MIC is the broth dilution method. Different concentrations of the agent are incubated with a known inoculum, and the minimum concentration which inhibits growth is determined. This can be measured turbidimetrically, and the determination can be performed using a microtitre plate, thus aiding automation of the process (317,318). These methods can also be used to demonstrate the spectrum of activity of the agent.

Both agar diffusion and broth dilution methods have been used to characterise the activity of new antibiotics against Pseudomonas aeruginosa and other ocular pathogens (158,163,183-191,199-209,213,317).

The bactericidal action of serum on Gram negative bacteria has been characterised by phase contrast (301), and electron microscopy (303).

The aim of this section of the project was to characterise the action of Anti-LPS by electron microscopy.

5.2 Materials and Methods

A large volume of nutrient broth was inoculated with Pseudomonas aeruginosa , and incubated overnight. This was then sub-cultured in fresh broth and incubated for a further 24 hours. The cells were collected by centrifugation, and washed twice with sterile saline. A heavy saline suspension, containing 1X10.10 organisms/ml, was used as the inoculum. Two centrifuge tubes were set up, containing:

1. 2ml inoculum
   4ml sterile saline
2. 2ml inoculum
   4ml Anti-LPS plasma.

They were incubated at 25oC for 5 minutes. The suspension was then centrifuged at 4000rpm for 5 minutes. The supernatant was removed, and the pellets of Pseudomonas cells were fixed and prepared for electron microscopy as follows (by the staff of the Ellis Rosenberg Electron Microscopy Unit, University of Natal Medical School):

1. pellet resuspended in 0,05M Karnovsky's fixative for 60 minutes.

2. washed in cacodylate buffer (twice, 10 minutes each).
3. post-fixed in 1% osmium tetroxide in sodium cacodylate buffer; pH 7,4; 4oC; for 60 minutes.
4. dehydrated through ascending grades of ethanol.
5. embedded in Araldite resin.

At each stage during this processing schedule, the cells were centrifuged at 4000rpm for 5 minutes to form a pellet. Ultrathin sections of approximately 70nm were cut with glass knives, using a Reichert 'Ultracut' ultramicrotome. The resulting sections were floated onto distilled water and mounted on uncoated copper grids. Sections were double stained in 1% ethanolic uranyl acetate and Reynold's lead citrate. Electron micrographs of the material were obtained with a Zeiss EM 10B electron microscope.

5.3 Results

Saline treated cells (control) : typical cylindrical bacilli, with an average lenghth of approximately 1,5um and an average diameter of

approximately 0,5um, were seen (Figures 6,7). An outer cell wall (+- 14nm) and an inner cell membrane (+- 7nm) could be distinguished. A number of cells of greater electron density could be seen. At higher magnification these were seen to be surrounded by an amorphous electron dense layer, +- 100nm thick (Figure 8).

Anti-LPS treated cells : low power micrographs showed fewer cells than the controls, with a far greater intercellular distance (Figure 9). Various morphological abnormalities, consistent with established theories of antibody-mediated bactericidal action, could be seen. Figure 10 shows a cell with early separation of the cell wall and cell membrane. The nuclear and cytoplasmic components of the cell still appeared normal. Figure 11 shows a typical spheroplast. The cell membrane was disprupted in a number of places, and the integrity of the cell was maintained by the single outer membrane (LPS containing layer) of the cell wall. The intracellular components appeared pale, flocular, and intermingled. Figure 12 shows a number of membrane ghosts, which appear to be the

remnants of lysed cells. An increased flocularity of the intercellular space was observed. A few of the more electron dense, encapsulated cells were still visible. These cells seemed unaffected by exposure to Anti-LPS plasma, but were surrounded by a border of proteinaceous material, external to the capsular material (Figure 13).

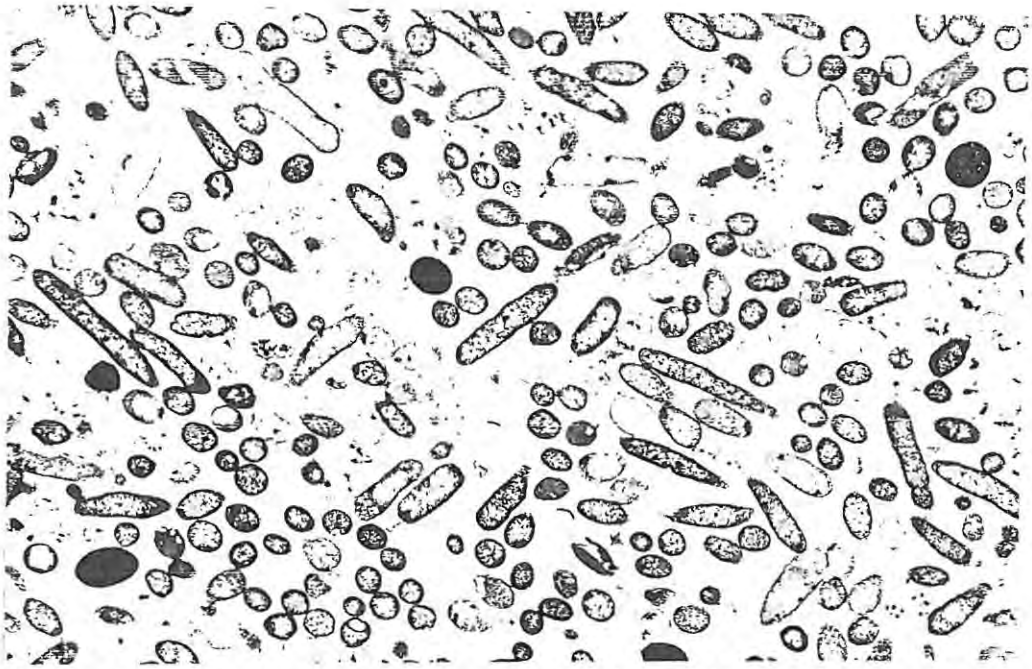

Figure 6: Control cells. Typical cylindrical bacilli of varying electron density (X 6 480).

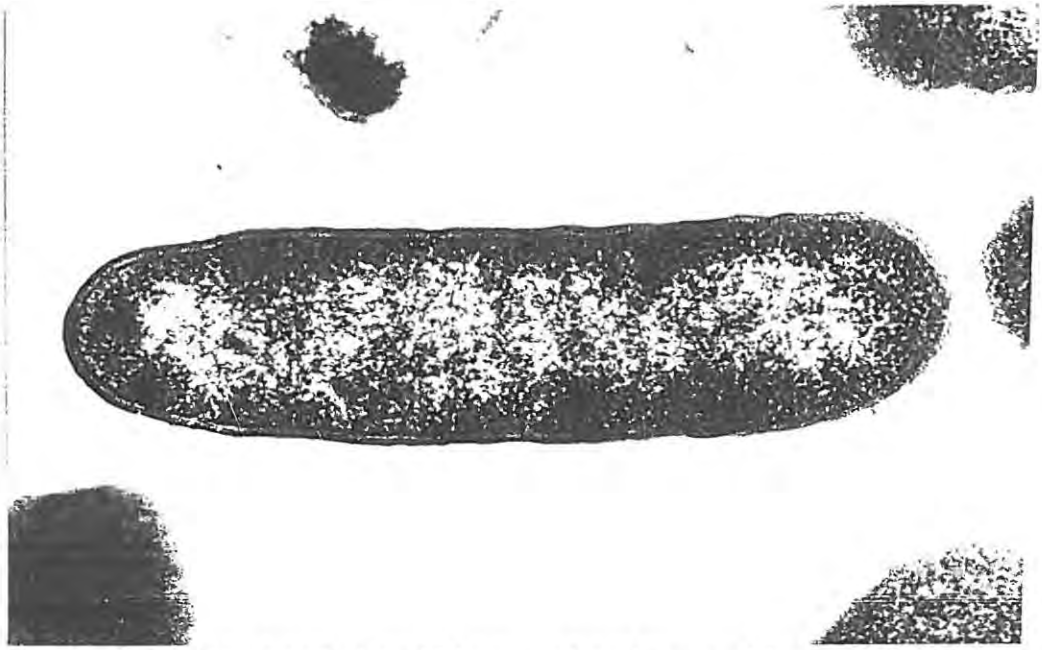

Figure 7: Typical bacillus, showing cell wall and cytoplasmic membrane systems (X 64 800).

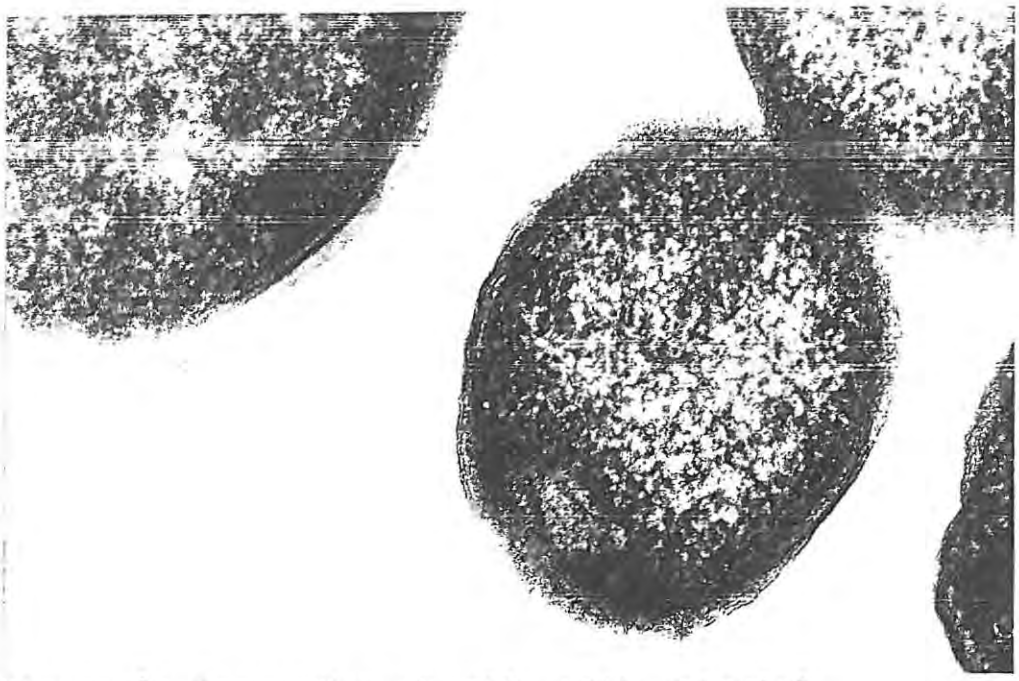

Figure 8: Encapsulated cell, with material external to the outer cell wall (X 128 000).

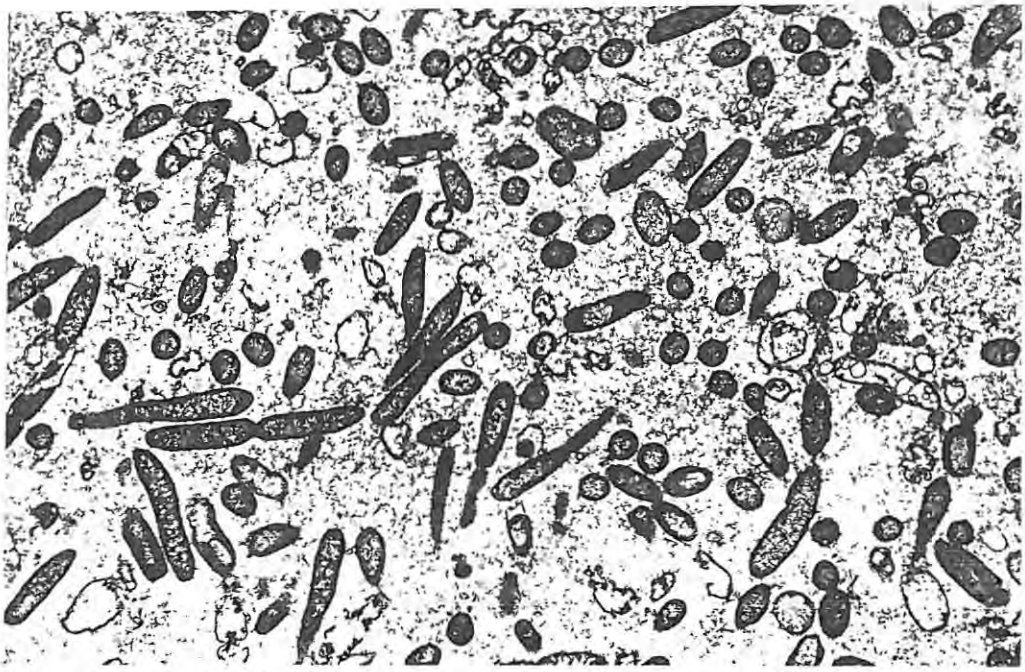

Figure 9: Anti-LPS cells, with fixed cell debris filling the intercellular spaces (X 6 480).

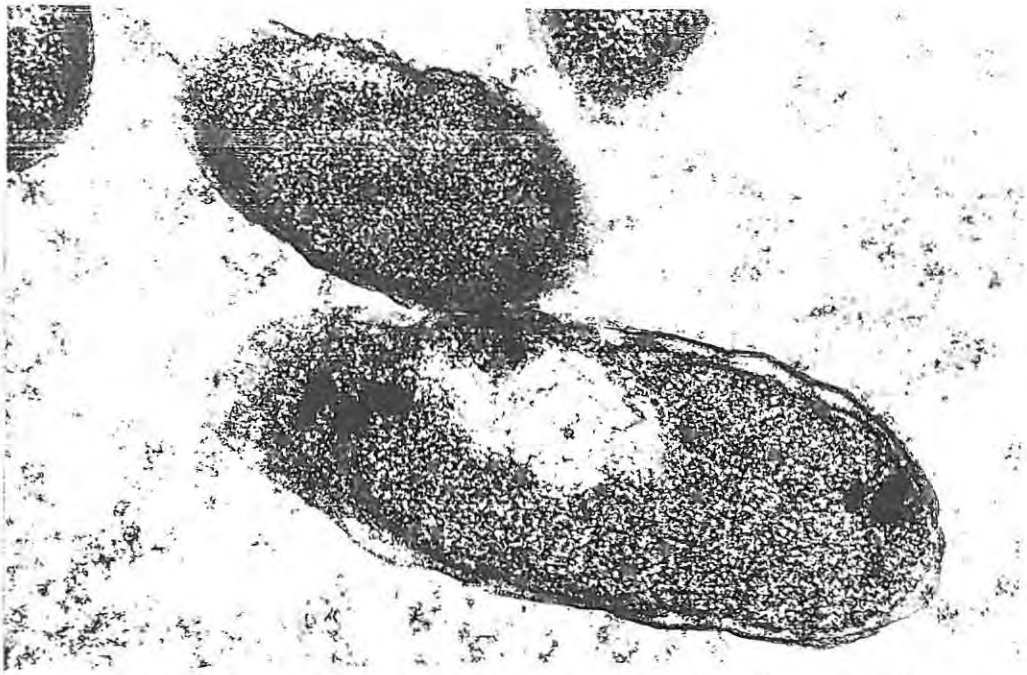

Figure 10: Treated cells showing early damage to the cell wall, with separation of the cytoplasmic membrane (X 64 800).

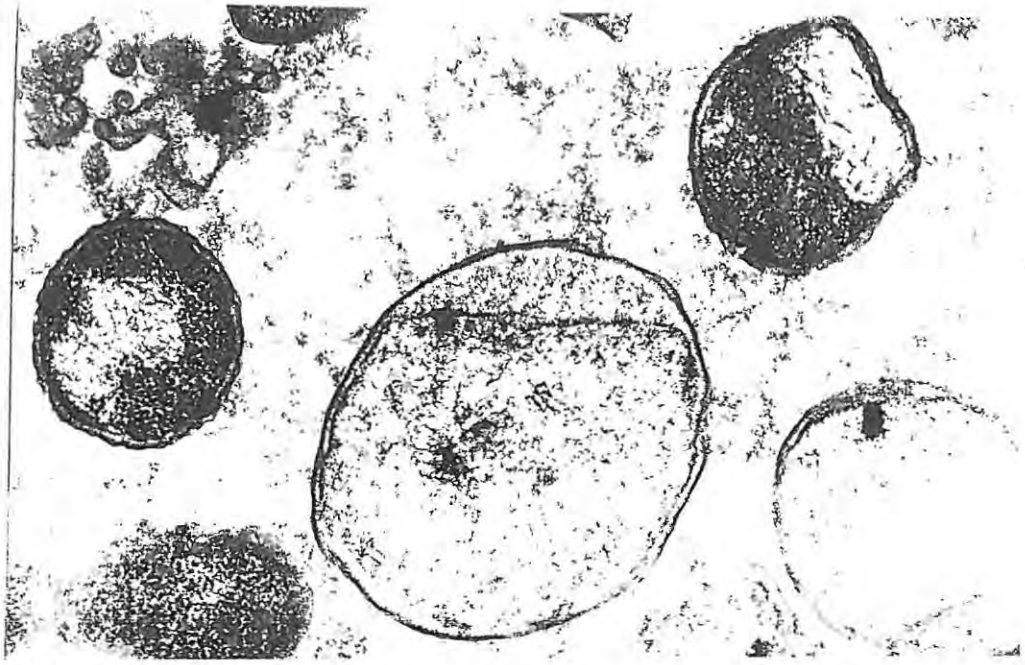

Figure 11: Spheroplast, showing total disruption of the cytopplasmic membrane (X 50 400).

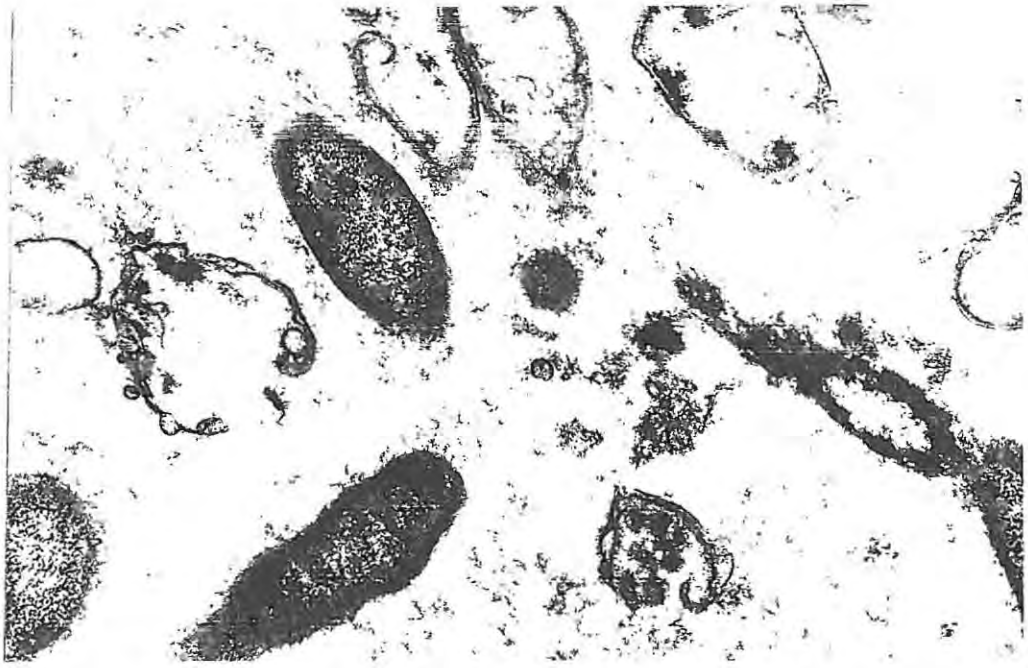

Figure 12: Membrane ghosts and cell debris, with more electron dense cells unaffected (X 32 480).

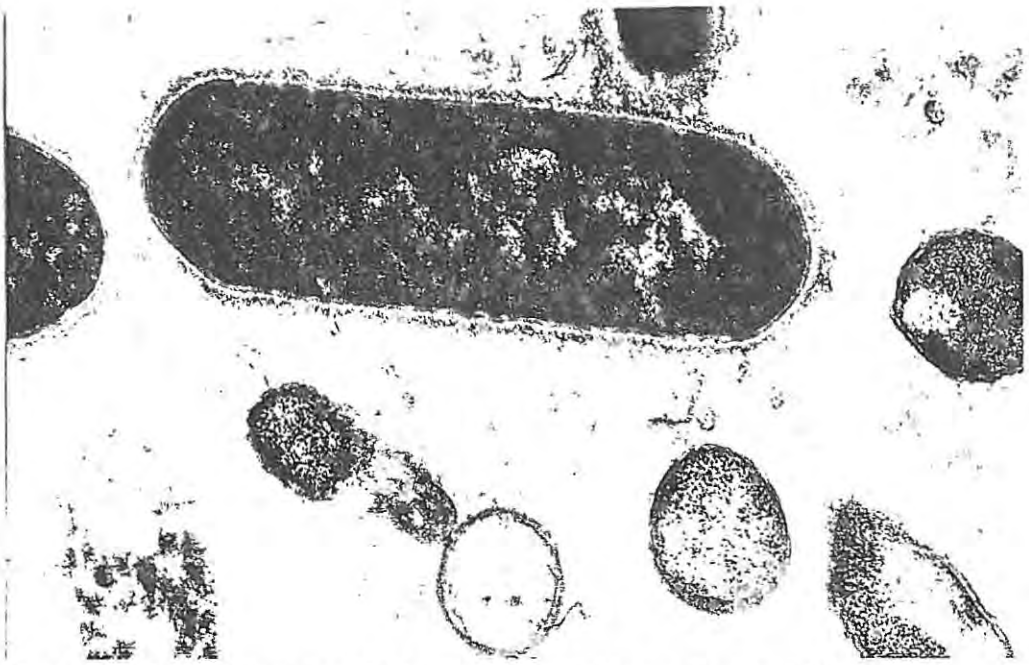

Figure 13: Encapsulated cell after Anti-LPS treatment, showing material adhering to the capsule (X 40 270).

5.4 Discussion

The direct bactericidal action of Anti-LPS plasma was clearly demonstrated by electron microscopy. Low power micrographs of plasma treated cells showed a reduced number of cells, with a far greater intercellular distance, compared to saline treated control cells. The intercellular space contained fixed proteinaceous material, presumably cellular debris resulting from plasma- induced cell lysis. The various stages of antibody-induced bactericidal action could be seen. The primary

site of damage is the bacterial cell wall, the antibody receptor being the 'bound' LPS component of the wall. Initially, damaged cells retained their normal cylindrical shape, but the cell wall and cytoplasmic membrane appeared to have separated in parts. This first stage is mediated by antibody and complement (301,302). Both the cell wall and the inner cytosplasmic membrane may be damaged by this antibody-complement system (303). Bactericidal activity is lost if complement is inactivated (304). Studies in this laboratory with human serum, rich in anti-LPS antibodies, have also shown that complement is required in this system (Gaffin, unpublished data). Serum heated at 56oC for 30 minutes lost the ability to agglutinate Klebsiella cells. When guinea pig complement was added, this ability was restored.

The second stage involves the conversion of the rod-shaped bacillus to a spherical form, a spheroplast. In some cases this is thought to be due to the enzymatic action of lysozyme on the damaged cell wall (301). In the absence of lysozyme, spheroplast formation did not occur, but

the rod-shaped cells remained non-viable (302). Pseudomonas spheroplast formation could only be demonstrated after 4 hours exposure to a very high concentration of carbenicillin. An extremely sensitive strain, with a low MIC for carbenicillin was used (319). In contrast, exposure of only 5 minutes was sufficient to demonstrate spheroplast formation due to the action of Anti-LPS plasma. The formation of Klebsiella spheroplasts has been demonstrated by Gaffin et al (unpublished data), using human anti-LPS serum. The average volume of these cells was shown to increase from 0,54um3 (normal controls) to 1,08um3. The outer cell wall still preserved the integrity of the cell, but the cytoplasmic membrane was disrupted.

The final event in the bactericidal process is probably due to osmotic effects (301,302). The cell wall of the spheroplast bursts, expelling the cytoplasmic contents. It is this cellular debris which probably prevented the surviving cells from approaching more closely during centrifugation and subsequent fixation. The addition of 0,5M sucrose to the medium has been shown to prevent this

bacteriolysis, by preventing the osmotic imbalance responsible for water entering the damaged cell (303).

A number of 'encapsulated' bacterial cells were seen in both the control and test samples. These bacterial cells appeared to be resistant to the bactericidal action of Anti-LPS plasma. Plasma treated cells were seen to be 'coated' with a proteinaceous material. It is possible that this was surface binding of antibodies, as shown for IgG raised against Braude's E.coli J5 vaccine (320). In that case, opsonization would still occur. The major role of natural antibodies in the defence against Pseudomonas invasion and infection is that of opsonization, largely because of the prevalence of serum resistance among clinical strains of Pseudomonas (77). This type of serum resistance has been found in E.coli 'smooth' strains (302). So-called 'smooth' strains have increased amounts of 'O' polysaccharide, which protects the inner 'rough' antigenic sites from antibody-complement attack. Mucoid strains of Pseudomonas, with increased amounts of exopolysaccharide, have been

shown to resist both antibiotics, and phagocytosis by tissue macrophages (321). Such mucoid strains are characteristically found in cystic fibrosis patients, where considerable damage is caused by such infection (322,323).

Anti-LPS plasma was therefore shown to exert a direct, rapid bactericidal action in vitro, as visualized by electron microscopy. However, no quantitative measure of this activity could be determined. The efficacy of this product in vivo isn dependent on a number of mechanisms, not only the direct bactericidal properties demonstrated here. Anti-LPS also opsonizes bacteria, enhancing phagocytosis, and exerts a direct antitoxic effect. These are not measured in the classic agar diffusion assays. The dominant role played by diffusion in this type of assay prevented any comparison of Anti-LPS and antibiotic potency, as measured by zone of inhibition size.

CHAPTER 6

6. GENERAL DISCUSSION AND CONCLUSIONS

6.1 Discussion of results

The impetus for this project was provided by the success achieved in using Anti-LPS plasma in a localised manner to treat various Gram negative infections (260,265). In particular, intra-uterine lavage successfully treated several Klebsiella infections of the uterus in stud mares. Previous work in the field of immunotherapy has been exclusively aimed at boosting the humoral immune system, by either active or passive immunization (226). The topical use of such products provides a new approach to the therapy of certain surface infections, such as Gram negative infections of the cornea.

Pseudomonas aeruginosa has emerged as an important nosocomial pathogen among debilitated, burned, and immunocompromised patients (6,324) It is also an important cause of infection in the military sphere

(6). This applies equally to ocular infections (4). Conventional antibiotic therapy of all Pseudomonas infections is hampered by the compromised state of the patient, and by the prevalence of resistance to antibiotics, especially amongst nosocomial strains (150). The frequent failure of such therapy has necessitated the use of alternative measures, such as immunotherapy. As early as 1927, Jackson et al. (325). recognized the potential of immuno-protection against Pseudomonas keratitis, even though only one good result was obtained in their experimental series. They concluded that "favourable results in this case suggest that an effective immune serum culd be produced". Vaccination with OEP, protease, and elastase toxoids (234), and with extracellular slime from mucoid strains (242). has effectively protected against experimental Pseudomonas keratitis. Hirao and Homma also used antisera against OEP, elastase and protease, in combination with a kanamycin-type antibiotic. They claimed that this combined antiserum-antibiotic regimen was effective therapeutically. However, the mice in this experiment were immunized 18 hours prior to

inoculation, and also received an IM injection of dibekacin just prior to the infection being induced. This amounts to a prophylactic regimen, and not therapy of established infections. To date no topical immunotherapeutic agent has been investigated. The aim of this project was thus to test the safety and efficacy of Anti-LPS plasma therapy for Pseudomonas keratitis in experimental animals. It was also hoped to quantify and characterise this action in vitro.

Once the suitability of this product for ocular administration had been demonstrated (vide 3.3), the first task was to establish a reproducible animal model of Pseudomonas keratitis. It proved extremely difficult to establish a progressive infection in rabbit eyes by the conventional needle scarification method. However, useful results were obtained in those rabbits in which such infections were established. The incidence of infection 'take' was similar to that reported by previous workers (274). As the effect of corticosteroids on the healing process was also to be examined, these agents were not used to increase the susceptibilty of the rabbits to the strain of Pseudomonas used.

A more intensive inoculation method was therefore considered. Hessburg's contaminated suture technique proved effective in all the rabbits so inoculated (276). An alternative laboratory animal, the guinea pig, was also used. The guinea pig was more susceptible to inoculation by needle scarification, and a consistent infection was established in all the eyes so inoculated.

Topical Anti-LPS therapy was effective in both the rabbit and guinea pig, when compared to saline lavages. The morbidity of the test and control eyes was comparable at the beginning of the trials, as measured by the 10-point grading system (Table 1). A statistically significant improvement was noted after Anti-LPS therapy (Table 4), and also when corticosteroids were added to this regimen (Table 9). The improvement noted was reflected in all three clinical parameters measured. This improvement was due to the specific properties of the plasma. The beneficial effects of the lavage were countered by the use of saline controls receiving the same physical treatment.

The individual variations in the response to

Anti-LPS prevented any accurate determination of a dose-response relationship for this type of treatment. Likewise, no accurate measure of the MIC of the plasma for the Pseudomonas strain used, could be determined in vitro. As this was a topical regime, aimed at a surface infection, attainment of MIC levels at the site of action was not a factor to be considered.

As the safety of this product for both topical and sub-conjunctival ocular administration has been clearly demonstrated, further research should now be directed at testing its safety and efficay in human clinical trials. In this way, by individualization of tne regimen, an optimum dosage may be found.

6.2 Pharmaceutical considerations

The plasma used in this study was collected by as clean a method as possible. Being a heat labile product, sterilization by ultrafiltration or X-irradiation are the only feasible methods. When used parenterally, the plasma was prepared

aseptically by this method (vide 2.5). Unit doses were packed, to minimise the chances of contamination, and eliminate the adverse effects of repeated freezing and thawing (326). Various preservatives are presently used in such biological products. Preliminary work in this laboratory indicates that the addition of 0,1% thiomersal can significantly prolong the shelf life of this product, if kept frozen. This preservative is known be well tolerated and is used extensively in human pharmaceuticals (327,328).

Various factors affect the penetration of topically applied medicaments into the tissue of the eye and into the aqueous humour (329): 1. molecular size 2. partition coefficient 3. degree of plasma binding. The size of the IgG molecule would prevent large scale penetration, although the inflamed state of the infected eye would ensure greater permeability (330-334). The absence of an effective epithelial barrier would also enhance the chances of antibody penetration. Davis et al (1978) (335) found that removal of the epithelium enhanced the efficacy of low dose topical

tobramycin therapy in a guinea pig model. The vital function of drug bioavailability was well stated by Benson (333) : " The susceptibilty of a disease process to a given drug means nothing, however, unless the drug reaches the site of the desired pharmacologic activity, and drug concentration sufficient for pharmacologic action is achieved at this site for the duration of treatment of the disease". Prolonged contact may be achieved by increasing the viscosity of the product, i.e., formulation as a semi-solid dosage form. At present an Anti-LPS 'paste', containing 8% sodium carboxymethylcellulose, is being tested in this laboratory, in a guinea pig Pseudomonas burn wound sepsis model. This type of vehicle has been used extensively in ophthalmology (333).

Plasma and saline lavages were administered via a simple tubing system in this trial. Various systems have been used to deliver large volumes of medicaments or irrigating agents to the eye. Hessburg (336) developed a sub-palpebral lavage system, but this is a severe and uncomfortable technique requiring surgical intervention. A more convenient method is the recently described

irrigating eyelid speculum (337). This hollow speculum has 3 irrigating portals in each arm, and can be connected to a syringe or infusion bottle. An irrigating contact lens has also been used by this group at Cullen Eye Institute, Houston Texas (Wilhelmus, pers. comm.). Contact lenses have also been used as drug delivery systems by being coated with or soaked in the antibiotic (44,223). However, the buildup of toxic Pseudomonas exoproducts is a problem in this type of therapy. Advantages include the protection of the ulcer from lid movement, and therefore from damage (splinting effect), and an increased antibiotic effect.

6.3 Ophthalmic applications for Anti-LPS therapy

The cross-reactivity of antibodies directed at certain LPS antigens has permitted the use of various immunogens to raise antibodies with a broad spectrum activity against Gram negative bacteria (78,297,338). This would enable Anti-LPS to be used for the therapy of ocular infections with other Gram negative bacteria, such as Proteus (339), Shigella(340), Serratia (341-344), and Moraxella (345). Moraxella bovis is a particular

(pers. comm. Dr KR Wilhelmus, Cullen Eye Inst., Baylor College of Medicine, Houston, Texas, USA)

problem in the veterinary field, causing infectious bovine keratoconjunctivitis (IBK) in certain breeds of cattle (346,347). Already, the use of immunological methods has been proposed as the only effective means of combating this disease (348). The cost of this disease in Australia, in terms of lost production, is estimated at more than $22 million per annum (346). A laboratory model of IBK has been described (349), and could form the basis for continued research using Anti-LPS plasma. Pseudomonas keratitis is also a problem in veterinary medicine (350).

Alternative routes of administration may enable Anti-LPS to be used for the therapy of other Pseudomonas infections of the ocular area, which resist current chemotherapy, eg., corneoscleral ulcers (351), scleritis (352), orbital cellulitis (353). Eye injuries, which may easily become infected with Pseudomonas, are particularly prevalent in the sphere of military medicine (23,24). Gram negative bacteria as a whole, and Pseudomonas in particular, are a common cause of infection in all war wounded (354-356).

Immunological methods of prophylaxis appear to be the most promising answer to these problems.

The prophylactic potential of Anti-LPS was not tested in this project. Current and previous work in this laboratory has demonstrated the protective capacity of Anti-LPS in a number of disease models (263). I have performed preliminary experiments using mice immmunocompromised by X-irradiation. They were treated prophylactically with sub-cutaneous injections of Anti-LPS, and then inoculated with Pseudomonas by the needle scarification method. No reduction in infection rate or extent was observed. Further work is needed to investigate this possibility more fully. The use of plasma lavages following ocular surgery might be of benefit in this regard.

6.4 Conclusions

Anti-LPS plasma was found to be a safe topical therapeutic agent, rapidly bactericidal for Gram negative bacteria in vitro, and effective in vivo against Pseudomonas keratitis in various

experimental models. Resistance to this agent has not been encountered, in this and other trials, and is not expected, because of the multitude of binding sites and mechanisms of action involved in its antibacterial activity. Human clinical trials are needed to further develop Anti-LPS as a useful adjunct to current antibacterial chemotherapy.

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APPENDIX A

The following computer programs were used, as described earlier (vide 2.7)

```
10 DISP "                    CHI SQUARE"
20 DISP
30 DISP "               FOURFOLD TABLE ANALYSIS"
40 DISP
50 DISP
60 DISP "   DO YOU WANT INSTRUCTIONS?  (1 = YES; 2 = NO)"
70 INPUT M
80 IF M=2 THEN 180
90 DISP " THIS IS A CHI SQUARE ANALYSIS OF THE FREQUENCIES OF EVENTS IN TWO POP
LATIONS"
100 DISP "YOU MUST ENTER AS REQUIRED THE NUMBER OF EVENTS IN POPULATION 'A' AS
ELL AS THE TOTAL POPULATION OF  'A'. DITTO POPULATION 'B'."
110 DISP
120 DISP "THE OUTPUT WILL BE: 1) THE DECIMAL FREQUENCIES OF THE EVENTS IN 'A' A
D 'B' AND 2)A DECISION WHETHER THESE FREQUENCIES ARE STATISTICALLY THE SAME"
130 DISP "OR DIFFERENT, AND TO WHAT PROBABILITY."
140 DISP
150 DISP
160 DISP "PRESS 'CONT' WHEN READY"
170 PAUSE
180 DISP "ENTER THE NUMBER OF POSITIVE EVENTS OF POPULATION 'A'"
190 INPUT A1
200 DISP "ENTER THE TOTAL POPULATION OF 'A' (THE DENOMINATOR FOR DETERMINATION
F FREQUENCY)."
210 INPUT A3
220 DISP "ENTER THE NUMBER OF POSITIVE EVENTS OF POPULATION 'B'."
230 INPUT B1
240 DISP "ENTER THE TOTAL POPULATION OF 'B'."
250 INPUT B3
260 A2=A3-A1
270 B2=B3-B1
280 C1=A1+B1
290 C2=A2+B2
300 C3=A3+B3
310 XSQ=(A1*B2-A2*B1)^2*C3/(A3*B3*C2*C1)
320 DISP " THE FREQUENCY OF 'A' (";A1;"/";A3;") IS";A1/A3*100;"%."
330 DISP
340 DISP " THE FREQUENCY OF 'B' (";B1;"/";B3;") IS";B1/B3*100;"%."
350 DISP
360 DISP " CHI SQUARE IS";XSQ
```

```
370 DISP
380 IF XSQ<.455 THEN 500
390 IF XSQ<= 1.31 THEN 520
395 IF XSQ<= 1.641 THEN 525
400 IF XSQ<= 2.69 THEN 540
410 IF XSQ<= 3.83 THEN 560
420 IF XSQ<= 5.01 THEN 580
430 IF XSQ<= 6.62 THEN 600
440 IF XSQ<= 7.87 THEN 620
450 DISP " ** THE TWO FREQUENCIES ARE STATISTICALLY DIFFERENT AT THE 0.1% LEVEL
(P<.001)."
451 DISP
460 DISP " IF YOU WANT A PRINTOUT PRESS 'CONT'"
470 PAUSE
480  GOTO 650
490 END
500 DISP " THE TWO FREQUENCIES ARE NOT STATISTICALLY DIFFERENT AT THE 50 % LEVEL
."
510 END
520 DISP " THE TWO FREQUENCIES ARE STATISTICALLY DIFFERENT ONLY AT THE 25% LEVEL
."
521 DISP
522 DISP "           YOU SHOULD TRY TO USE A LARGER SAMPLE SIZE."
523 END
525 DISP " **  THE TWO FREQUENCIES ARE  STATISTICALLY DIFFERENT ONLY AT THE 20%
LEVEL."
526 DISP
527 DISP "           YOU SHOULD TRY TO USE A LARGER SAMPLE SIZE."
530 END
540 DISP "* THE TWO FREQUENCIES ARE STATISTICALLY DIFFERENT AT THE 10% LEVEL (P<
.10)."
550 END
560 DISP "*THE TWO FREQUENCIES ARE STATISTICALLY DIFFERENT AT THE 5% LEVEL.(P<.0
5)."
570 END
580 DISP "* THE TWO FREQUENCIES ARE STATISTICALLY DIFFERENT AT THE 2.5% LEVEL (P
<.025)."
590 END
600 DISP " THE TWO FREQUENCIES ARE STATISTICALLY DIFFERENT AT THE 1% LEVEL (P<.0
1)."
610 END
620 DISP " THE TWO FREQUENCIES ARE STATISTICALLY DIFFERENT AT THE 0.5% LEVEL (P<
.005)."
630 DISP " IF YOU WANT TO GET A PRINTOUT PRESS 'CONT'"
640 PAUSE
650 PRINT " THE TWO FREQUENCIES ARE STATISTICALLY DIFFERENT AT THE 0.1% LEVEL (P
<.001)."
660 END
```

```
10 DISP "                         'T' TEST"
20 DISP
30 DISP "                  DIFFERENCES BETWEEN MEANS"
40 DISP
50 DISP
60 DISP "THIS PROGRAM DETERMINES WHETHER THERE IS A SIGNIFICANT DIFFERENCE BETWI
EN THE MEANS OF TWO DIFFERENT GROUPS OF NUMBERS."
70 DISP
80 DISP "     IT CALCULATES THE 'T' VALUE OBTAINED WHEN BOTH GROUPS OF NUMBERS
RE ENTERED INTO THE COMPUTER.  THIS 'T' VALUE MUST THEN BE COMPARED TO "
90 DISP "MATHEMATICAL TABLES OF 'T' VALUES TO DETERMINE WHETHER DIFFERENCES IN
EANS ARE SIGNIFICANT, AND TO WHAT PROBABLILITY."
100 DISP
110 DISP "                PRESS 'CONT' BUTTON"
120 DISP
130 PAUSE
140 DISP
150 DISP "     YOU MUST ENTER THE NUMBER OF DATA POINTS IN BOTH SETS OF NUMBERS
(THEY NEED NOT BE EQUAL) WITH BETWEEN 3 AND 1000 POINTS IN EACH SETS."
160 DISP
170 DISP "     YOU MUST ENTER THE DATA POINTS OF X, AND THEN OF Y, BY PUTTING TH
E NUMBERS IN THE DATA LINE APPROX. 370 AND 460."
180 DISP
190 DISP
200 DISP
210 DISP
220 DISP
230 DISP "                PRESS 'CONT' BUTTON"
240 DISP
250 DISP
260 DISP
270 PAUSE
280 DISP
290 DISP
300 DISP "ENTER THE NUMBER OF POINTS OF DATA SET 'X' ."
310 INPUT NX
320 DISP "ENTER THE NUMBER OF POINTS OF DATA SET 'Y' ."
330 INPUT NY
340 DIM X(1000),Y(1000)
350 FOR I=1 TO NX
360 READ X(I)
370 ! THE FOLLOWING DATA ARE OF 'X'
380 DATA
440 DISP "X(";I;") = ";X(I)
450 PRINT "X(";I;") = ";X(I)
460 NEXT I
470 FOR I=1 TO NY
480 READ Y(I)
490 ! THE FOLLOWING DATA ARE OF 'Y'
500 DATA
510 DISP "Y(";I;") =";Y(I)
515 PRINT "Y(";I;") =";Y(I)
520 NEXT I
530 X1=0
540 FOR I=1 TO NX
550 X1=X1+X(I)
560 NEXT I
570  DISP
580 DISP "X1 =";X1
590 Y1=0
600 FOR I=1 TO NY
610 Y1=Y1+Y(I)
```

```
620 NEXT I
630 DISP "Y1 = ";Y1
640 DISP
650 XBAR=X1/NX
660 DISP "MEAN X  += ";XBAR
670 PRINT "MEAN 'X' =";XBAR
680 YBAR=Y1/NY
690 DISP "MEAN Y =";YBAR
700 PRINT
710 PRINT "MEAN 'Y' =";YBAR
720 ! NOW TO CALCULATE THE SUMS OF DIFFERENCES SQUARED."
730 SSIGX2=0
740 FOR J=1 TO NX
750 SIGX2=(X(J)-XBAR)^2
760 SSIGX2=SSIGX2+SIGX2
770 NEXT J
780 STDDEVX=(SSIGX2/(NX-1))^.5
790 DISP "STD DEV OF 'X' IS";STDDEVX
800 PRINT
810 PRINT "STD DEV 'X' =";STDDEVX
820 SSIGY2=0
830 FOR J=1 TO NY
840 SIGY2=(Y(J)-YBAR)^2
850 SSIGY2=SSIGY2+SIGY2
860 NEXT J
870 STDDEV=(SSIGY2/(NY-1))^.5
880 DISP "STD DEV OF 'Y' IS";STDDEV
890 PRINT "STD DEV 'Y' IS";STDDEV
900 T=(XBAR-YBAR)/((SSIGX2+SSIGY2)*(1/NX+1/NY)/(NX+NY-2))^.5
910 DISP
920 DISP " T = ";T
930 PRINT
940 PRINT " 'T' = ";T
950 PRINT
960 DISP "     ++++++++  TABLE OF T VALUES+++++++++++++++  "
970 DISP
980 DISP "  DEGREES    **********PROBABILITY*************** "
990 DISP "  FREEDOM     20%     10%      5%       2%      1%   "
1000 DISP "  ---------------------------------------------------"
1010 DISP "      1      3.078   6.314   12.702   31.821   63.657"
1020 DISP "     10      1.372   1.812    2.228    2.764    3.169 "
1030 DISP "     30      1.310   1.697    2.042    2.457    2.750  "
1040 DISP "   INFINITY 1.281   1.644    1.960    2.326    2.575  "
1050 DISP "-----------------------------------------------   "
1060 PRINT
1070 PRINT "++++++++++++  TABLE OF T VALUES+++++++++++++++  "
1080 PRINT
1090 PRINT "DEGREES    **********PROBABILITY*************** "
1100 PRINT " OF"
1110 PRINT "FREEDOM     20%     10%      5%       2%      1%   "
1120 PRINT "---------------------------------------------------"
1130 PRINT
1140 PRINT "    1      3.078   6.314   12.702   31.821   63.657"
1150 PRINT "   10      1.372   1.812    2.228    2.764    3.169 "
1160 PRINT "   30      1.310   1.697    2.042    2.457    2.750  "
1170 PRINT "INFINITY 1.281   1.644    1.960    2.326    2.575  "
1180 PRINT
1190 PRINT
1200 PRINT
1210 PRINT
1220 PRINT
1230 END
```

APPENDIX B

The following papers on this work were accepted for publication:

Topical immunotherapy for Pseudomonas keratitis in rabbits: use of antilipopolysaccharide plasma
by NH Welsh, AJ Rauch, and SL Gaffin
(British Journal of Ophthalmology)

Topical immunotherapy with Anti-LPS plasma: use in aguinea pig model of Pseudomonas keratitis
by AJ Rauch, NH Welsh, and SL Gaffin
(Ark)

TOPICAL IMMUNOTHERAPY FOR PSEUDOMONAS KERATITIS IN RABBITS : USE OF ANTI-LIPOPOLYSACCHARIDE PLASMA*

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SUMMARY

Pseudomonas keratitis is currently treated with antibiotics with a variable success rate. Part of the morbidity caused by Pseudomonas is due to the action of lipopolysaccharide (LPS) present on the surface membrane of the bacteria. Specific IgG present in equine Anti-LPS hyperimmune plasma has been found to bind to the LPS from a range of gram negative bacteria, including Pseudomonas and, by activating complement, destroys these bacteria. Anti-LPS plasma was therefore used as a therapeutic agent in experimentally induced Pseudomonas keratitis in rabbits. Thirteen out of fifteen (86,7%) Anti-LPS treated eyes improved whereas four out of seventeen (23,5%) saline treated control eyes improved ($X^2 = 12,76$ $p < 0,001$). No ill-effects were noted when Anti-LPS was administered to healthy rabbit or baboon eyes. Anti-LPS thus was protective in Pseudomonas keratitis, and clinical trials appear to be warranted.

Pseudomonas aeruginosa is responsible for severe keratitis that may progress to panophthalmitis. It is particularly disastrous when it occurs as a post-operative complication of invasive surgery, e.g. penetrating keratoplasty. Current therapy is based on antibiotics which exhibit antipseudomonal activity, such as gentamicin and tobramycin[1]. High systemic doses are required to obtain adequate intra-ocular drug levels. The use of these agents is associated with a high incidence of side effects such as ototoxicity and nephrotoxicity[2].

The pathophysiology of Pseudomonas infections involves a number of components and products of the bacterial cell[3]. Enzymes are produced which have a destructive effect on the host cell, e.g. proteases and elastases. The lipopolysaccharide (LPS, endotoxin) component of the cell wall has also been implicated in the disease[4,5]. This has been shown to be the cause of non-infectious corneal ring formation, mediated by complement activation[6,7]. Antibiotics exert a direct antibacterial effect only, and thus immunotherapy directed at other aspects of the condition has enjoyed increased attention[8].

Gaffin and colleagues have developed an equine Anti-LPS hyper-immune plasma containing specific IgG which binds to free LPS and also has bactericidal properties[9,10]. This plasma has been used successfully to treat a variety of Gram-negative infections involving LPS in horses, dogs, cats, sheep and rats[11]. The diversity and cross-reactivity of the antibodies in the preparation enable it to kill a wide range of Gram-negative bacteria, including Pseudomonas, Klebsiella, E. coli, Proteus, Shigella and Salmonella. Such serum or plasma has recently proved effective in the prophylaxis and therapy of endotoxaemia in the veterinary field, particularly the race horse industry of South Africa[12].

This study has been conducted to determine whether Anti-LPS hyperimmune equine plasma (Anti-LPS) would be effective in treating corneal Pseudomonas infections. Anti-LPS was first tested against in vitro cultures of Pseudomonas aeruginosa, and found to be bactericidal. The serum was then found safe to use topically on normal rabbit eyes, as a lavage. The Anti-LPS was then used therapeutically on experimentally-induced Pseudomonas keratitis in rabbits.

MATERIALS AND METHODS

An untyped culture of Pseudomonas aeruginosa was obtained from a Microbiology Department of a University Hospital. It was cultured in nutrient broth for 24 hours, to a final concentration of 600 - 900 million organisms/ml.

Anti-LPS was prepared from plasmapheresed horses, suitably immunized (ATOX Pharmaceutical Co., 14 Old Main Road, 3600 Gillitts, South Africa). The plasma contained 1500 ug/ml of LPS precipitible antibodies. These antibodies could bind to endotoxins prepared from Sh. flexneri, 5 strains of E. coli, 5 species of Salmonella, Klebsiella, Proteus and Pseudomonas.

Rabbits of mixed, non-inbred strain, weighing 2,5 - 3,5 kg were used. All had normal corneas and anterior segments. The corneas were anaesthetised locally with 3 drops of oxybuprocaine H·Cl prior to inoculation.

Method of inoculation

Two methods were used to show that Anti-LPS was effective in both moderate (Group A) and severe infections (Group B).

Group A: Both corneas of rabbits were prepared by making 3 deep vertical and horizontal incisions into the stroma, in a cross-hatch pattern, using a sterile 20 gauge needle, according to Furgiuele [13]. The epithelium was removed in a 5 x 5 mm area. Four drops of the bacterial inoculum were dropped onto the cornea, and allowed to remain in contact with the cornea and inferior fornix for 30 seconds.

Group B: Rabbit eyes were infected with contaminated sutures[14]. A virgin silk suture (8/0) was soaked for 5 minutes in a broth culture of Pseudomonas. The suture was then passed through the corneal stroma. The ends were cut, and the sutures were allowed to remain in the cornea for 2 days. The lids were not sutured together.

Following inoculation the animals were examined daily. The eyes were photographed at the commencement and end of treatment and the lesions were assessed on slit-lamp biomicroscopy. The severity of the infection present was graded according to the following parameters:

a) area of cornea involved

b) depth of lesion

c) degree of vasqularization

This sytem was modified from that of previous workers [15,18]. Each grade varied from 0 to 3 points. Thus (a)0 indicated no infection, (a)1 up to 33% corneal area involved, (a)2, 33 to 66%, (a)3, 66 to 100% affected. If the opacification was nebulous, the score was (b)0, if it was macula (b)1, if it was moderate lecuomatous, (b)2, severe lecuomatous(b)3. If the

cornea perforated, this was denoted as (b)3p. Finally, if no vascularization was present, it would be (c)0, if mild (c)1, if moderate (c)2, if severe, (c)3. Therefore a moderate keratitis involving 50% of the cornea, with mild opacity, and some vascularization would be scored as (a)2 (b)1 (c)1. The most severe stage would be (a)3 (b)3p (c)3. In assessing overall severity, the points were added together, so that a maximum of 9p was obtainable. (Table 1).

A masked randomized trial was initiated. The rabbits were treated as follows. One infected eye received Anti-LPS, and where possible (infections established in both eyes) the contralateral eye served as a control, receiving saline. Anti-LPS or saline was administered as a lavage, at the rate of 40 drops/minute, for 5 minutes, 3 times a day. The treated and control eyes were chosen at random. Treatment started as soon as the infection was clearly established, i.e. after 2 days. The final appearance of the eyes was evaluated after 8 days, by which time, Anti-LPS had neutralised the effect of the Pseudomonas.

Since we wished to determine merely whether the serum could control and limit the infection, the final result would not necessarily be a reversal of the lesions, but a quiet eye with scar formation. In 5 severe cases, topical corticosteroid drops (dexamethasone disodium phosphate) were added to this regimen after 8 days, as it has been reported that steroids can cause a recurrence of the infection [16,17]. Steroids were also administered to 5 control eyes.

RESULTS

In Group A rabbits, an infection developed in 22 out of 32 eyes inoculated. This developed in 2 days or less, and varied

in severity. In Group B a severe infection developed in 10/10 eyes inoculated.

Table 2 shows the results of treatment with Anti-LPS, compared to saline controls. Fifteen infected eyes were treated with Anti-LPS, and 13 (86,7% X^2 = 12,76, $p < 0,001$) improved, with arrest of the keratitis and some with partial healing. Of the 17 saline treated infected eyes, 4 (23,5%) improved, 9 deteriorated and 4 remained unchanged. The unchanged eyes were already in the most severe group, so further deterioration could only have led to perforation.

Table 3 shows the changes that occurred on the 1st, 4th and 8th day. Five out of 17 saline treated control eyes in Group A and B perforated by day 8, 4/17 improved spontaneously, 4 remained unchanged. Among Anti-LPS treated eyes, only 1 deteriorated, and 1 was unchanged.

Table 4 shows the complete scores on day 1 and day 8.

Figure 1A shows the eye of rabbit 7 before treatment, and Figure 1B shows the same eye after 8 days of Anti-LPS treatment. Five serum-treated eyes that received topical corticosteroid after the 8th day showed dramatic improvement, and 3 of the control eyes continued to deteriorate. These are not indicated in the tables.

DISCUSSION

Our experimental model was similar to previous models to assess the different modes of treatment for Pseudomonas keratitis [13,14]. Where possible the Anti-LPS treated eye was compared with the contralateral eye of the same animal treated with saline. The

eyes were allocated for treatment in a randomized fashion. Additionally, assessment was conducted by outside observers, to eliminate bias.

Anti-LPS treatment resulted in significant improvement in the infected eyes. IgG present in the Anti-LPS binds to LPS located on the outer cell membrane of Pseudomonas. This activates complement, and causes the lysis and destruction of bacteria. This specific IgG also binds to the LPS released from the killed bacteria, in part neutralizing it. Moreover, such binding enhances host phagocytosis of the 'free' LPS. Treatment in this experimental series was only initiated when the infection was firmly established. In 10/15 eyes, the plasma was used on eyes severely infected, i.e. morbidity indices of 6 or more, and the eyes responded after 3 - 4 days. The improvement in the corneal infection involved all 3 parameters measured, area of keratitis, depth of lesion, and degree of vascularization.

In milder cases the plasma was effective with reversal of the lesion, whereas in severe cases the plasma effectively brought the infection under control, and the final result was a firm scar (Table 3). It would be advantageous to initiate therapy at the earliest sign of clinical infection, but in this experimental analysis, we wished to determine the plasma's efficacy against established infections.

The purely physical action (lavage) of instillation of solutions on the corneal surface has been shown to be beneficial by removing necrotic tissue and the exoenzymes produced by Pseudomonas[14]. Therefore saline was administered in the same manner to the control eyes. It would thus seem that the dual action of Anti-LPS (bactericidal and antitoxic) is effective in Pseudomonas keratitis. Significantly, no eyes in the Anti-LPS treated group perforated. Bohigian et al has shown that, in contrast to the 'melting' character of Pseudomonas keratitis in humans, the condition in

rabbits follows an ulcerative, necrotic course, such that the addition of collagenase inhibitors to the normal regimen had no significant effect on the clinical course[18].

We believe that the addition of Anti-LPS could significantly change the corneal stromal reaction. In another study, using carbenicillin and gentamicin, it was found that the keratitis continued to worsen for up to 7 days before improvement was noted[19]. In a different experimental model Anti-LPS has already been observed to be more effective therapeutically than gentamicin. In our study with Anti-LPS, improvement commenced within 3 days. Experiments in progress show that Anti-LPS is also effective in similar infections in guinea pigs.

The dosage regimen used was arbitrary (40 drops/min x 5 minutes t.i.d. x 8 days). We have yet to determine whether this may be modified or simplified. Treatment by the sub-conjunctival route will also be investigated, as preliminary results indicate that this method is tolerated by the normal rabbit eye.

A possible complication of Anti-LPS treatment could be a systemic reaction to foreign equine antigens. However, this has not occurred in other experimental models where Anti-LPS has been used, or in veterinary clinical practice[11,12].

The addition of topical corticosteroid after 8 days resulted in an additional dramatic decrease in superficial vascularization, and a reduction in the area of scarring. The serum treatment does not therefore contraindicate subsequent use of steroids, and indeed this seems to be beneficial.

We believe that the use of Anti-LPS in the treatment of Pseudomonas keratitis in rabbits shows favourable results, with none of the disadvantages of conventional antibiotic therapy, and that it has a potential application in the therapy, and perhaps prophylaxis, of the human condition.

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Table 1: Method of scoring severity of experimental Pseudomonas keratitis

% surface of cornea affected	grade (a)	density and depth	grade (b)	vascular-ization	grade (c)
0	(a) 0	none	(b) 0	none	(c) 0
1 - 33	(a) 1	mild macula	(b) 1	mild	(c) 1
34 - 66	(a) 2	moderate leucomatous	(b) 2	moderate	(c) 2
67 - 100	(a) 3	severe leuco-matous perforation	(b) 3	severe	(c) 3

Maximum score totally all factors a + b + c = 9

Table 2: Treatment of Pseudomonas Infected Eyes

Treatment	No. of eyes treated	Improved (%)	Unchanged (%)	Deteriorated (%)
Saline	17	4 (23,5)	4 (23,5)	9 (52,9)
Anti-LPS	15	13 (86,7) *	1 (6,7) **	1 (6,7) ***

* $X^2 = 12,76$ $p < 0,001$

** $X^2 = 1,72$ $p < 0,1$, N.D.

*** $X^2 = 7,94$ $p < 0,001$

Table 3: Morbidity Index of Corneas*

		Controls (normal saline)				Treated			
	Rabbit No.	1	4	8	Results**	1	4	8	Results
GROUP A	1	9	8	9	0	9	9	7	+
	2	9	9	9	0	9	9	7	+
	3	9	9	9p	-	4	6	6	-
	4	5	2	2	+	5	4	4	+
	5	3	2	2	+	9	6	4	+
	6	9	9	9	0	4	2	2	+
	7	6	8	9	-	9	8	6	+
	8	6	8	9	-	5	4	3	+
	9	6	8	9	-	6	6	4	+
	10	6	4	3	+				
	11	6	8	9	-				
	12	3	2	2	+				
GROUP B	13	8	9	9p	-	8	7	6	+
	14	8	9	9p	-	8	8	6	+
	15	8	9	9p	-	8	8	6	+
	16	8	9	9p	-	8	7	6	+
	17	8	8	8	0	9	9	9	0

* Summation of scores (0 - 3 of a) area of keratitis, b) depth of lesion and c) vascularization. p indicates performation of cornea.

**+ = improvement
0 = no change
- = deterioration

Table 4: Clinical evaluation of Pseudomonas keratitis before and after treatment

		CONTROL						TREATED EYE					
		1st day			8th day			1st day			8th day		
		surface (a)	depth (b)	vessels (c)	surface (a)	depth (b)	vessels (c)	surface (a)	depth (b)	vessels (c)	surface (a)	depth (b)	vessels (c)
GROUP A	1	3	3	3	3	3	3	3	3	3	2	3	2
	2	3	3	3	3	3	3	3	3	3	2	2	3
	3	3	3	3	3	3p	3	1	2	1	2	2	2*
	4	2	2	1	1	1	0*	2	2	1	1	2	1
	5	1	1	1	1	1	0*	3	3	3	1	2	1
	6	3	3	3	3	3	3	1	2	1	1	1	0
	7	2	2	2	3	3	3	3	3	3	2	3	1
	8	2	2	2	3	3	3	2	2	1	1	1	1
	9	2	2	2	3	3	3	2	2	2	2	1	1
	10	2	2	2	1	1	1*	1	2	1	1	1	0
	11	2	2	2	3	3	3						
	12	1	1	1	1	1	1*						
GROUP B	13	3	2	3	3	3p	3	3	2	3	2	2	2
	14	3	2	3	3	3p	3	3	2	3	2	2	2
	15	3	2	3	3	3p	3	3	2	3	2	2	2
	16	3	2	3	3	3p	3	3	2	3	2	2	2
	17	2	3	3	3	2	3	3	3	3	3	3	3*

* indicates controlled eyes that improved

* indicates treated eyes that deteriorated or did not improve

3p indicates perforation

TOPICAL IMMUNOTHERAPY WITH ANTI-LPS PLASMA : USE IN A GUINEA PIG MODEL OF PSEUDOMONAS KERATITIS *

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ABSTRACT

Pseudomonas infections of the cornea present serious problems in therapy. Part of the morbidity is mediated by lipopolysaccharide (LPS) present in the outer surface of the infecting Gram negative bacteria. Equine hyperimmune antilipopolysaccharide plasma (Anti-LPS) has previously been found to be therapeutic in Pseudomonas and other Gram negative bacterial infections in animals and man. We here administered Anti-LPS topically to Pseudomonas-infected guinea pig corneas. Of the saline treated control eyes, only 1/11 (9,1%) improved, while of the Anti-LPS treated eyes, 7/11 (63,6%) (X^2 = 7,07; $p < 0,005$) improved. Clinical trials appear warranted to further develop this therapeutic regimen.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic, Gram negative pathogen responsible for severe, sight-threatening infections of the human cornea (keratitis). Current chemotherapy frequently fails because of the ability of the organism to become resistant to the anti-bacterial agent used[1]. An equine hyperimmune plasma, containing high concentrations of LPS-specific IgG antibodies, has been used by Gaffin and coworkers to treat a variety of Gram negative infections[2]. This plasma inactivates and opsonizes LPS and, in addition, may destroy the parent bacteria by complement activation[3]. We have previously found that Anti-LPS is therapeutic in Pseudomonas infections of the rabbit cornea[4]. This study was conducted in order to determine whether topical administration of Anti-LPS would be effective in treating Pseudomonas keratitis in a guinea pig model.

MATERIALS AND METHODS

Eleven pigmented, non-inbred guinea pigs were anaesthetised with ether. They were then inoculated by the following procedure: A 20-gauge needle was dipped in a 24 hour nutrient broth culture of Pseudomonas aeruginosa, and was used to make 2 non-penetrating horizontal scratches in the central cornea. An area of ca. 2 mm x 2mm was removed from the central epithelium.

This procedure consistently caused severe corneal infections in 12 hours.

Treatment was initiated after 18 hours. One eye of each guinea pig received Anti-LPS*, at a rate of 40 drops/minute for 5 minutes, 3 times a day. The contralateral control eye received the same treatment with an equal volume of sterile saline.

We previously found that in an untreated guinea pig, the infection progressed until day 7, being worst at day 5[5,6]. At this stage the corneal infection was still suppurative, and presented as a totally infiltrated, opaque, vascularized eye. Thereafter the infection subsided, and the eye was usually "quiet" by day 14, with a dense, leukomatous scar. The appearance of the eyes was therefore evaluated on day 7 of treatment. Treated eyes were described as being either "improved" or "not improved". Eyes described as "improved" had no discharge at day 7, compared to controls, and healed with only nebulous scarring, compared to the dense scars on the control eyes.

RESULTS

A consistent infection was produced in all 22 eyes inoculated. The results are shown in Table 1.

Only 1/11 (9,1%) of the saline treated control eyes improved significantly in discharge or area and density of scarring. However, 7/11 (63,5%) of the Anti-LPS treated eyes improved (X^2 = 7,07; $p < 0,005$), although none cleared completely. Many of the Anti-LPS treated eyes healed, showing only minimal, nebulous scarring, with return of the red reflex (i.e. the pupil and retina were visible under the slit-lamp biomicroscope).

Figure 1 shows the eye of guinea pig 4, before (A) and after (B) Anti-LPS treatment.

*obtained from Pharmaceutical Co., 14 old Main Road, Gillitts, Natal

DISCUSSION

Anti-LPS treatment resulted in a significant improvement in Pseudomonas-infected eyes by the seventh day. A difference between treated and control eyes was still evident after 14 days, even though the infection in guinea pigs is characteristically self-limiting. This improvement can be attributed to the specific properties of Anti-LPS (antitoxic and bactericidal), as well as to the physical effects of the lavage administration, removing necrotic tissue and Pseudomonas exoproducts[7].

These and other results in our laboratory[4] indicate that Anti-LPS immunotherapy is potentially useful as an adjunct to conventional therapy for Pseudomonas surface infections such as keratitis. Clinical trials are warranted since previous studies have already demonstrated the safety of Anti-LPS for topical ocular administration.

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TABLE 1 : Treatment of Pseudomonas keratitis in guinea pigs

TREATMENT	NUMBER OF EYES TREATED	IMPROVED (%)
Saline	11	1 (9,1)
Anti-LPS	11	7 (63,6)*

* $X^2 = 7,07$; $p < 0,005$

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