THE USE OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE DETERMINATION AND CHARACTERIZATION OF ANTIENDOTOXIN ANTIBODIES

by

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Submitted in partial fulfilment of the requirements
for the degree of Master of Science (Med.Sci.) in the
Department of Physiology,
University of Natal
1984

Durban 1984

PREFACE

The experimental work described in this thesis was carried out in the Department of Physiology, University of Natal, Durban, from February 1981 to March 1983, under the supervision of Dr. Stephen L. Gaffin.

These studies represent original work by the author and have not been submitted in any form to another University. Where use was made of the work of others it has been duly acknowledged in the text.

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to the following who have made the presentation of this thesis possible:

Dr S.L. Gaffin, Senior Lecturer in the Department of Physiology, University of Natal, for initiating the study, for his excellent supervision of the thesis and for his constant encouragement and assistance.

Dr. J.D. Conradie and Mr. B.J. Vorster of the Natal Blood Transfusion Service, Pinetown, for their valuable assistance and collaboration in this study.

Professor J.G. Brock-Utne, Head of the Department of Physiology, University of Natal, for allowing me full use of facilities in his department.

Finally my husband Omar and daughter Farzanah for their patience and encouragement which made the completion of this thesis possible.

ABSTRACT

Recent clinical studies have highlighted the effectiveness of immunotherapy for Gram-negative bacteraemia in humans. Studies in America, undertaken on patients with Gram-negative bacteraemia, have shown that mortality was reduced by virtually 50% in patients who received specific antiendotoxin antiserum. In India, mortality from pseudomonas septicaemia was significantly reduced by the administration of small quantities of a anti-pseudomonas immunoglobulin.

The antibodies in those studies were raised by vaccination of healthy volunteers with heat-killed Gram-negative bacteria or vaccines containing endotoxin. Adverse side effects in volunteers as well as logistic and legal problems make it difficult to produce antiserum on a large scale, in this manner.

In Israel, S.L. Gaffin and coworkers found that approximately 7% of plasma units in a blood bank had antiendotoxin antibody concentrations of 40 µg/ml or greater. This high titre human plasma significantly protected cats from lethal endotoxic shock secondary to haemorrhage. The immunoprecipitin technique used by them to measure antiendotoxin antibody concentrations was unsuitable for screening large numbers of blood samples. To overcome this problem we have devised an enzyme-linked immunosorbent assay (ELISA) for determining the level of antiendotoxin immunoglobulin G in human plasma. The assay, which is suitable for large scale use, was found to be specific for antiendotoxin antibodies. It was calibrated using a serum sample of specific antibody concentration as determined by an immunoprecipitin assay.

Serum samples found to be high in antiendotoxin titres (> 40 µg/ml) were tested for their specificity towards endotoxins from 12 bacterial

strains and species. While each sample was found to have its own characteristic specificities, most were found to react strongly with Sh. flexneri, S. typhimurium and S. enteritidis.

The Natal Blood Transfusion Service has found that in Natal, blood units containing high concentrations of specific antibodies occur with a frequency of 3,6% among all White donors and 10,35% among all African donors. They found that African females, in turn, had almost twice the frequency of high titre serum as African males. In this study, Indian female hospital patients did not have a statistically higher frequency of high-titre serum than Indian male patients.

Blood units donated to the Natal Blood Transfusion Service are now routinely screened by ELISA for antiendotoxin antibodies and those units with high concentrations (> 40 ug/ml) of antibody were pooled and fractionated to obtain a gamma globulin, Lot LG-1. The binding capacity of the LG-1 antibodies towards 12 endotoxins was examined. Binding was found to be highest with endotoxin from Sh. flexneri, S. abortus equi and S. typhimurium and intermediate with S. enteritidis and E.coli 026:B6. Binding with the other endotoxins tested was relatively low. Differential absorption experiments showed that LG-1 was made up of a mixture of cross-reacting as well as specific antibodies. For example, the antibodies binding Sh. flexneri endotoxin were mainly specific. Those binding E. coli 026:B6 endotoxin were specific and cross-reacting in almost equal proportions. Antibodies to the endotoxins from the salmonella strains tested were mainly cross-reacting. The specificities of the LG-1 antibodies towards endotoxins from the various Gram-negative bacteria did not in most cases reflect the incidence of these organisms in blood cultures taken from hospital patients.

The activity of LG-1 antibodies was compared to that of normal human immunoglobulin preparations obtained from the National Blood Fractionation Centre, Pinetown and to an anti-pseudomonas immunoglobulin prepared by Wellcome Laboratories, England. The binding capacity of the antibodies in the standard globulin preparations towards most of the endotoxins tested was less than 15% of that of the LG-1 antibodies. The anti-pseudomonas immunoglobulin was shown to bind poorly to most of the endotoxins tested in comparison with binding by LG-1 antibodies.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Endotoxin, also referred to as pyrogen or lipopolysaccharide (LPS) is a poison found as an integral part of the cell surface of Gram-negative bacteria[1]. Such bacteria and therefore endotoxin are always present and contained within the intestines. In normal individuals, small quantities of endotoxin leak into the portal circulation and are readily cleared by the reticuloendothelial system in the liver[2]. If however, the permeability of the intestines is altered or liver function is impaired, then endotoxaemia occurs, resulting in pathological changes which may ultimately culminate in shock and death. Endotoxin may also enter the circulation through a septic focus, resulting from, for example, wounds and burns. Patients whose defence mechanisms are compromised by diseases such as leukaemia and lymphoma or by treatment with immunosuppressive drugs are particularly susceptible to bacterial infections[3]. Even with the best available treatment, septicaemia is a major cause of death in patients with cancer[4].

In the United States, the frequency of Gram-negative bacteraemia has been estimated at approximately 1 per 100 hospital patients, with fatality rates of 30-50% and is thus a major health problem[5]. Different types of treatment have been used in an attempt to lower the mortality from Gram-negative bacteraemia. Despite the use of powerful antibiotics, high doses of adrenocorticosteroids, modern life-support systems and sophisticated monitoring devices, the incidence of bacteraemia continues to increase with sustained high mortality rates[6].

It has long been recognized that tolerance to some of the biological effects of endotoxin can be induced by vaccination of animals with

endotoxin, or killed Gram-negative bacteria[7,8]. Enhanced resistance to endotoxin is also found after infections with Gram-negative bacteria. Furthermore, the passive transfer of serum from endotoxin-tolerant animals is known to protect recipients against the lethal effects of endotoxin[9,10]. Fever, the local Shwartzman reaction, intravascular coagulation, hypotension and even death from endotoxin have been prevented in animals by the use of antiserum. Much of this work has been reviewed by Braude[11]. Specific antibody present in the antiserum is thought to be resposible for the inactivation of endotoxin.

In 1973, it was found that human antiserum could also confer passive immunity to the effects of endotoxin in animals[12]. In recent years clinical studies have highlighted the effectiveness of immunotherapy for Gram-negative bacteraemia in humans. Studies in America, undertaken in patients severely ill with Gram-negative bacteraemia, have shown that mortality was reduced by virtually fifty percent in those patients who received specific antiendotoxin antiserum[13-15]. In India, where the risk of death from pseudomonas septicaemia resulting from severe burns is high, it was found that mortality was very significantly reduced by the administration of small quantities of an anti-pseudomonas immunoglobulin[16].

These noteworthy studies have however some serious limitations. The antibodies were raised by vaccination of healthy volunteers with heat-killed Gram-negative bacteria or vaccines containing LPS[13-16]. Injection of humans with potentially dangerous substances even under controlled conditions is undesirable and is reported to have led to local and/or systemic reactions in some volunteers[17]. Thus, the side-effects in immunized volunteers as well as logistic problems make it difficult to produce antiserum on a large scale, in this manner.

As a way of overcoming these problems, S.L. Gaffin and coworkers in Israel, screened units of plasma in a blood bank to find samples rich

in antiendotoxin antibodies[18]. They found that approximately 7% of these units had antiendotoxin antibody concentrations of 40 µg/ml or greater. The high titre human plasma, after absorption of agglutinins, was used to treat cats who were in lethal haemorrhagic shock. Survival was markedly improved in those cats infused with the antiendotoxin antibody-rich plasma compared to that in controls.

The above work has been subsequently extended in South Africa in conjunction with the Natal Blood Transfusion Service. In the Israeli study, an immunoprecipitin assay was used to measure the antiendotoxin antibody levels in blood units. This technique is unsuitable for screening large numbers of blood samples on a routine basis since it takes two and a half days in order to complete the assay and only a few samples can be conveniently handled at a time. To overcome this problem we attempted to devise an enzyme-linked immunosorbent assay (ELISA) for determining the level of antiendotoxin antibodies in human plasma. The technique of ELISA, pioneered by Engvall and Perlmann[19-21] and Van Weemen and Schuurs[22] has been adapted for use with disposable microtitre plates providing an assay system which is convenient for large scale use[23]. It has the added advantage of being economical since only small volumes of reagents are required. The reagents are relatively cheap and stable, having a long half-life. ELISA compares very favourably in sensitivity to radioimmunoassay and gives results which may be determined visually or with simple photometers[23]. In the laboratories of the Natal Blood Transfusion Service, ELISA is used extensively to measure a range of antibodies and antigens, for example, serum ferritin, α-fetoprotein, anti-rubella, anti-tetanus etc[24].

Thus the purpose of this study was to develop an ELISA for the screening of human blood for antiendotoxin antibodies and to investigate the specificity of the antibodies according to their ability to react with various pure endotoxins obtained from several bacterial species which are of clinical importance.

CHAPTER 2
LITERATURE REVIEW

2.1 THE STRUCTURE OF THE GRAM-NEGATIVE BACTERIAL CELL WALL

Bacteria are divided into two major groups depending on their reaction to the Gram staining procedure[25]. Cells are stained with crystal violet and iodine and then washed with an organic solvent such as alcohol. The latter step decolourizes Gram-negative bacteria but not Gram-positive bacteria. These reactions, in turn, depend on the structure and chemistry of the cell walls.

Gram-negative organisms have a thin peptidoglycan layer adjacent to the outer surface of the cell membrane[26,27]. Other substances lie outside this peptidoglycan layer; lipoprotein serves to cross-link the peptidoglycan with an outer membrane. Associated with the outer membrane is the lipopolysaccharide which contributes most to the uniqueness of the Gram-negative cell wall. A diagrammetic representation of the Gram-negative cell envelope is given in Figure 1[25].

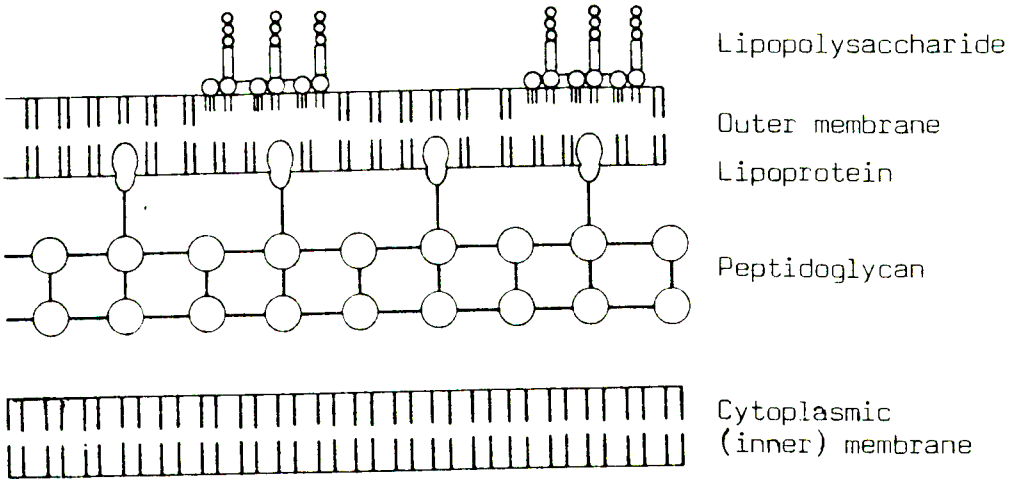


Figure 1. A model of the Gram-negative cell envelope[25].

Gram-positive organisms, such as the Staphylococci, Streptococci, Clostridia and Bacilli, have much thicker multi-layered peptidoglycan coats to which polysaccharides are covalently bound[28]. Complex polymers of teichoic acids make up 20 to 50% of the dry weight of the Gram-positive cell walls.

The lipopolysaccharide or endotoxin is thus only found in Gram-negative organisms, where it forms an integral part of the cell surface and as such it is mainly released when the cells are lysed[29]. Exotoxins on the other hand, are protein in nature and are secreted by living cells, both Gram-positive and Gram-negative[30]. Tetanus toxin, for example, is one of the most poisonous exotoxins known.

2.2 THE CHEMICAL STRUCTURE OF ENDOTOXIN

2.2.1 Introduction

Endotoxins from different groups of Gram-negative bacteria share a common structural basis[29]. These macromolecules, the molecular weights of which vary from 400,000 to 4,000,000 Daltons, are made up of three major components, lipid A, core polysaccharide and the "O" antigens.

The elucidation of the structure of lipopolysaccharides has been advanced by the use of rough (R) mutants that are defective in the synthesis of the complete lipopolysaccharide[31]. Such R mutants have been isolated from strains of various Gram-negative species including Escherichia, Salmonella and Shigella.

Many procedures have been developed for the extraction of endotoxin from bacterial cell walls. The most widely used techniques are adaptations of either the trichloroacetic acid (TCA) procedure of

Boivin and Mesrobeanu[32] or the hot aqueous phenol extraction procedure of Westphal and colleagues[33].

The TCA procedure yields complexes of endotoxin containing lipopolysaccharide and protein while the phenol procedure results in a relatively protein free preparation of LPS. Standard endotoxin preparations with well characterized biological and chemical properties are now commercially available and their use is intended to avoid problems with reproducibility among laboratories using endotoxins[34].

The structure of each of the three major components of lipopolysaccharide will be examined.

2.2.2 Lipid A

Lipid A has been isolated from mutant bacterial strains having endotoxins containing only lipid A and KDO (2-keto-3-deoxyoctonate), a trisaccharide of which normally links lipid A to the core region of endotoxin[31]. Preparations of lipid A are also obtained from lipopolysaccharides by acid hydrolysis, which cleaves the bonds between lipid A and KDO. Lipid A contains glucosamine, phosphate and long chain fatty acids. It is an unusual phospholipid in that glucosamine and not glycerol forms the core[29].

Salmonella lipid A, which has been extensively studied, has a backbone of a β1,6-linked D-glucosamine disaccharide, which is phosphorylated in positions 1 and 4'[1]. These phospate groups are partially substituted by 4-amino-4-deoxy-L-arabinosyl and phosphorylethanolamine residues. The backbone structure is illustrated in Figure 2, which also shows the numbering system for the sugars.

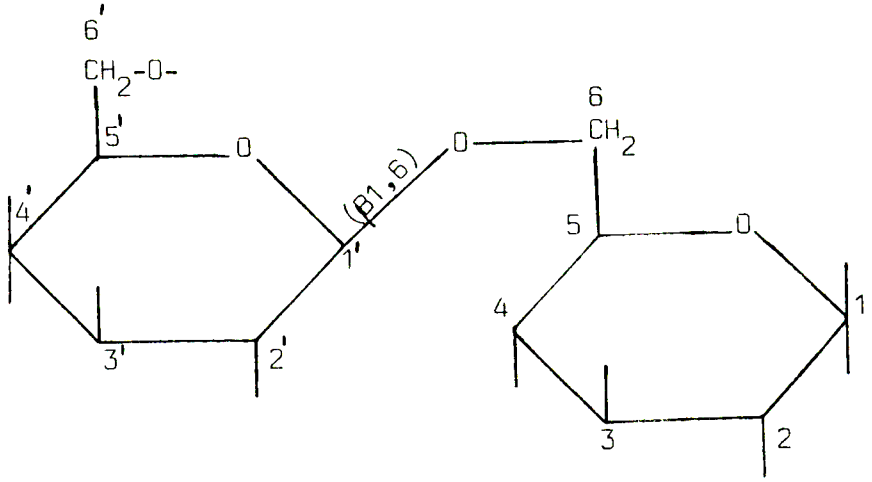


Figure 2. The glucosamine disaccharide structure, showing the β1,6 linkage and sugar numbering system.

The amino groups of the disaccharide are acylated by β-hydroxymyristic acid (3-hydroxytetradecanoic acid), which is a 14 - carbon fatty acid present specifically in enterobacterial lipid A as well as in Pseudomonas. β-hydroxy fatty acids containing between 10 and 17 carbon atoms are found in other bacterial species[29].

The hydroxyl groups at positions 3,4 and 6' of the glucosamine disaccharide are esterified by a range of long chain fatty acids, e.g. lauric, palmitic and 3-myristoxymyristic acid[29]. The disaccharide subunits are linked to each other through phosphodiester bridges or pyrophosphate linkages. Lipid A is associated with hydrophobic regions of the outer membrane and in this way binds the LPS to the cell membrane[26].

Lipid As extracted from a range of Gram-negative species have been

compared and found to share the basic structural features described above, with only limited variability; e.g., the types of fatty acids present[1]. Figure 3 from Lüderitz and coworkers illustrates the proposed structure of a lipid A unit of Salmonella minnesota R595[31].

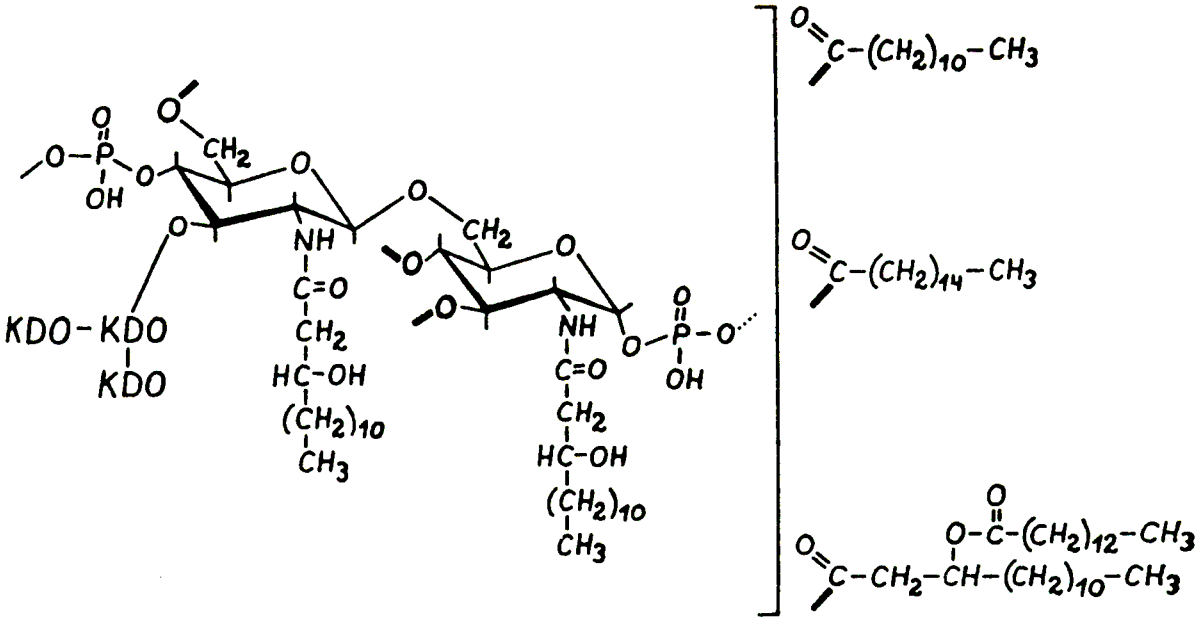


Figure 3. Proposed structure of a lipid A unit of the Salmonella minnesota R595 glycolipid with an attached KDO trisaccharide. The three fatty-acid residues shown are linked in an unknown distribution to the hydroxyl groups of the glucosamine residues available at positions 3, 4, and 6'[31].

2.2.3 Core polysaccharide

Lipid A is linked to the core region by means of a trisaccharide of KDO, 2-keto-3-deoxyoctonate[29]. Other components of the "core", from varying species and strains, include heptose, phosphate, ethanolamine and the hexoses, galatose, glucose and N-acetyl gluocosamine.

An inner core comprising KDO, heptose phosphate and ethanolamine is assembled and linked to the three hexoses which are said to form the outer core.

The core polysaccharide structure has been studied in different salmonella mutants which lack specific enzyme(s) so that synthesis of the core remains incomplete[29]. Chemotype Rd of salmonella LPS contains only KDO and heptose, while in chemotype Rc glucose is added to this backbone. In chemotype b galactose is further incorporated and finally the core of phenotype Ra is synthesized in full. This is summarized in Figure 4 below:

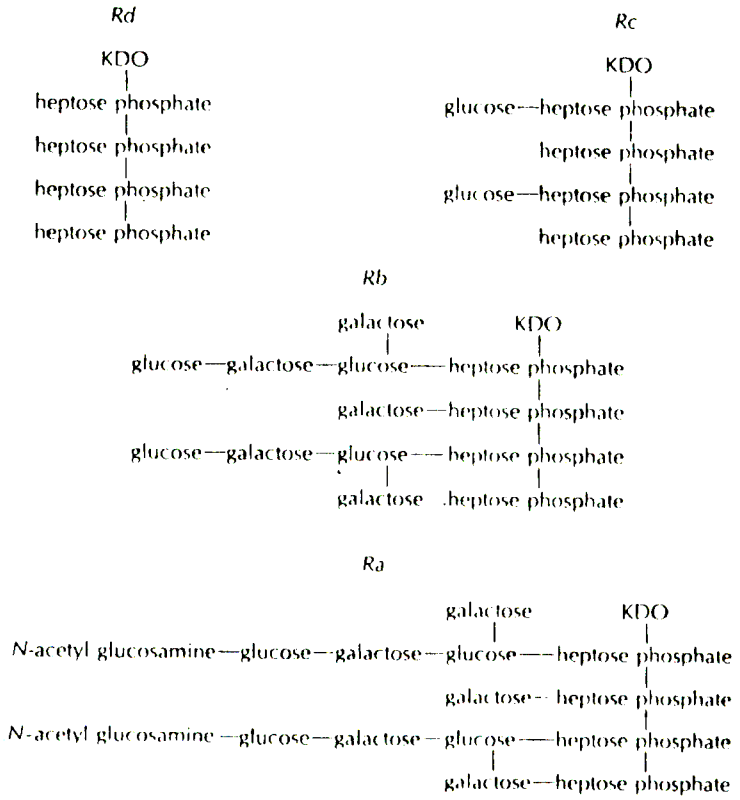


Figure 4. The core polysaccharides of chemotypes Rd, Rc, Rb and Ra of Salmonella LPS[29]

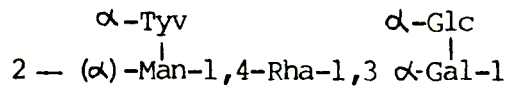
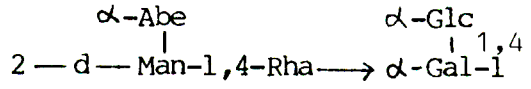
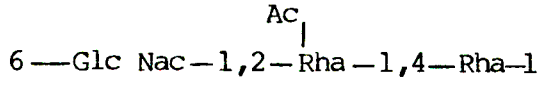
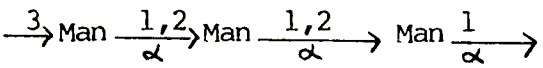
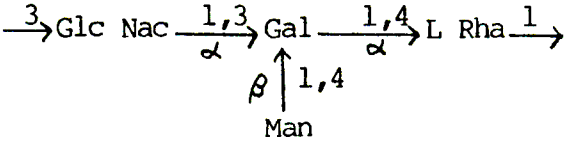
All salmonella species have been found to synthesize LPS with a polysaccharide core basically structured as described above. Some variability in the core structure of LPS from the other species has been reported[1]. However, this variability is low compared to that of the "O" side chains.

2.2.4 "O" antigens

The second polysaccharide component of LPS consists of the "O"-specific chains, which are polymers of oligosaccharide repeating units making up the outermost part of the LPS molecule[29]. These oligosaccharide units contain usually between 3 to 5 sugars each and often include relatively unusual sugars such a fucose, rhamnose, paratose, abequose, colitose and tyvelose. When LPS of different origin is examined much variability is found in the structure of the "O" specific chains[1]. The nature of the individual sugars, their sequence and the type of linkages and substitutions vary for LPSs from different bacterial strains. Table 1 shows the structure of the repeating oligosaccharide units in some Gram-negative bacteria.

Table 1

Composition of repeating oligosaccharide units in certain Gram-negative bacteria.

Bacteria	Structure of Oligosaccharide Unit	Group or Serogroup
Salmonella typhosa[29]		D
Salmonella typhimurium[29]		B
Shigella flexneri[29]		2a
Escherichia coli[35]		08
Escherichia coli[35]		075

Glc = glucose; Gal = galactose; Rha = rhamnose; Man = mannose;
Glc Nac = N-acetyl glucosamine; Abe = abequose; Tyv = tyvelose;
Ac = acetyl

2.2.5 Summary

In summary, Figure 5 diagrammatically represents the characteristic structural features of a typical salmonella LPS, showing the Lipid A, core polysaccharide and "O" antigens.

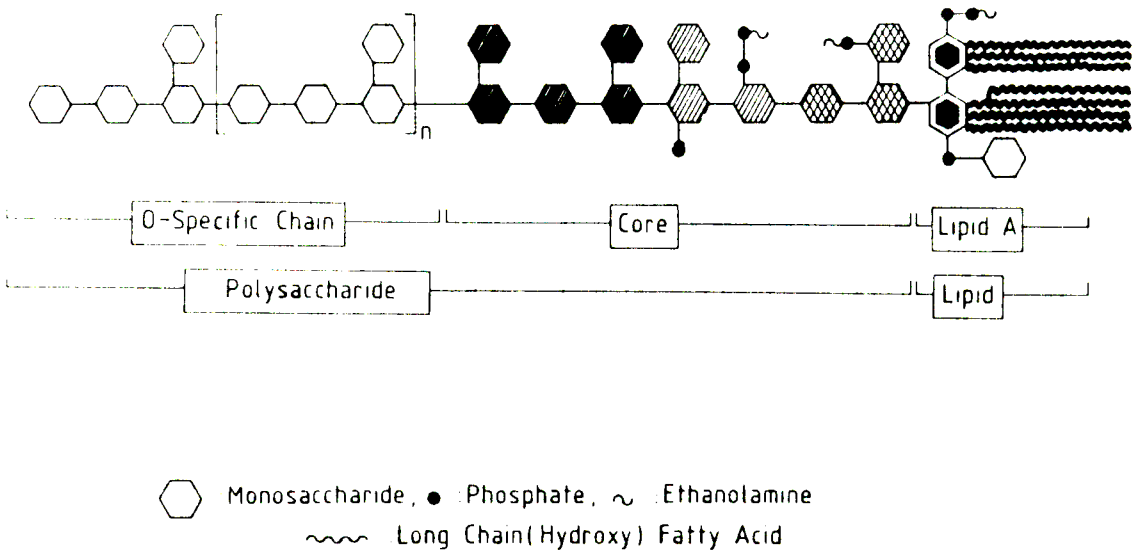


Figure 5. Schematic structure of a salmonella lipopolysaccharide[1].

The component of LPS which varies least in composition in different Gram-negative bacterial species is lipid A[1]. It is this region which has now been shown to be responsible for the toxicity of the LPS molecule. It is suggested that the toxic component is associated with the acylated central disaccharide.

Free lipid A has been tested for endotoxic activity by means of mouse lethality, pyrogenicity, bone marrow necrosis, Limulus gelation and complement inactivation studies[31]. Lüderitz and coworkers report that their results "strongly indicate that lipid A represents the biologically active centre in endotoxic lipopolysaccharides and that

the polysaccharide component acts as a solubilizing carrier that can be replaced by other non covalently bound carriers like serum albumin". Thus, the "O" antigens are not directly involved in the endotoxic activity but are thought instead to protect the bacteria from attack by host enzymes and from ingestion by host phagocytic cells. The protection is expected to be enhanced by the presence of relatively uncommon "exotic" sugars[36]. Lipid A is poorly antigenic while core polysaccharide is highly antigenic[11]. Nevertheless anti-lipid A antibodies have been measured in, for example, patients with urinary tract infections; the titres are said to be caused by immunogenically active lipid A in the body[37].

2.3 THE BIOLOGICAL EFFECTS OF ENDOTOXINS

The effects of endotoxins are widespread and varied. Some of the principal actions are outlined below.

2.3.1 Hypotension

High levels of circulating endotoxin cause a series of haemodynamic changes which ultimately lead to hypotension. These are summarized below.

(a) Endotoxin causes the release of vasoactive agents such as catecholamines, prostaglandins, histamine, serotonin and kinins[38-42].

(b) These vasoactive agents bring about the local contraction of neighbouring capillary endothelial cells away from each other forming "holes" in the capillary walls and directly damaging the cells leading to their vacuolisaton[43-46]. The pore size in the endothelium is altered and this subsequently allows the escape of plasma and red cells.

(c) This epithelial damage results in increased permeability to blood components and consequent oedema and hypovolaemia.
Endothelial damage in the lungs results in pulmonary oedema so that proper gas exchange is impared - "shock lung"[47-50].
Similarly, in the case of intestinal membranes, endothelial damage leads to gastrointestinal bleeding[48].

(d) This plasma loss leads to haemoconcentration with increased blood viscosity[51].

(e) Venous return is reduced. This is due to loss of plasma as well as to vasoconstriction of central and hepatic veins in turn leading to the accumulation of blood in the splanchnic region or "splanchnic pooling"[51,52]. However, Guntheroth and coworkers find that this hepatic trapping of blood seems in dogs to last only the first 30 minutes after intravenous injection of endotoxin[53].

(f) The above events lead to a reduction in cardiac output and subsequent hypotension. The fall in cardiac output results in reduced oxygen supply to the tissues[54-56]. Acidosis results with increased plasma lactate[55,57-59].

(g) Endogenous opiates are released by the action of endotoxins and may be responsible for further reducing blood pressure. The endogenous opiate β-endorphin is stored with pituitary adrenocorticotrophin, ACTH. Since endotoxins cause the secretion of ACTH, it is considered likely that endotoxin also causes release of pituitary β-endorphin at the same time[60]. Naloxone, a specific opiate-antagonist, is found to be effective in reversing endotoxin-induced hypotension in rats[61,62]. Naloxone is also reported to be effective in reversing haemorrhagic shock in cats[63].

Under the above conditions electrolyte balance is disturbed[64]. The concentration of potassium in the plasma is increased while intracellular levels are decreased. The intracellular concentration of sodium is increased.

All these effects contribute towards the deterioration of lysosomal membranes and subsequent release of proteinases and other hydrolytic enzymes, especially from the pancreas[52,65,66].

2.3.2 Pyrogenicity

The pyrogenic properties of endotoxins have been recognised for many years. The fever which results after intravenous injection of endotoxin is thought to be mediated by a protein, "endogenous pyrogen" released from neutrophils or monocytes upon activation by endotoxin[67]. Endogenous pyrogen in turn stimulates the temperature regulation centres in the hypothalamus[11,68]. Moreover, endotoxin itself appears to be capable of causing fever directly since when micro-injected into the anterior hypothalmic preoptic region, fever can be caused by a 100 times lower dose than when injected intravenously[69].

It has been suggested that humans are much more sensitive to the pyrogenic action of endotoxin than other animals, for example, the same febrile response in rabbits and humans with S. typhosa endotoxin requires 10 times as much endotoxin (0.05 µg/kg body weight) in the rabbit as in man (0.005 µg/kg body weight)[70]. Other studies have found rabbits to be equal or several times more responsive than man[67].

Detailed studies of fever after injection of endotoxin have been carried out in rabbits[29,67]. After intravenous injection of 0,1 µg endotoxin, there is a variable latent period of 10 to 20 minutes before the onset of a biphasic fever, with peaks at about 70 minutes

and at about 3 hours. The second peak alone is dose dependent and is not caused by smaller doses of endotoxin.

Skarnes and coworkers report endotoxin-induced biphasic fevers in sheep[71]. Assays of plasma obtained during fever episodes show a marked increase in prostaglandin E (PGE) during the initial phase of fever only. Indomethacin, an inhibitor of prostaglandin metabolism, is shown to block fever responses to both endotoxin and endogenous pyrogen. These results implicate PGE as the mediator of the first phase of endotoxin induced fever and also suggest that prostaglandins may be involved in mediating the second phase of fever, associated with circulating endogenous pyrogen.

2.3.3 Disseminated intravascular coagulation

The observations of Sanarelli and others of the effects of two injections of culture filtrates from Gram-negative organisms first demonstrated what is now known to be the ability of endotoxin to produce tissue injury through initiation of coagulative changes[72-75]. These early observations were extended and resulted in two widely used experimental models : the local and the generalized Shwartzman reactions.

In order to produce these reactions in experimental animals, two injections of endotoxin are given 12 to 18 hours apart[29]. For the local Shwartzman reaction the first dose is given intradermally and the second intravenously. Within a few hours after the second dose an area of haemorrhagic necrosis is seen at the site of the first injection.

For the generalized Shwartzman reaction both endotoxin injections are given intravenously. Within a few hours of the second dose, the animals develop bilateral cortical necrosis of the kidneys[44].

An intravenous dose of endotoxin causes intravascular coagulation and subsequent production of fibrin polymers. In the local Shwartzman reaction these polymers are trapped in skin vessels which have been injured by an inflammatory reaction caused by the intradermal dose of endotoxin. No renal cortical necrosis is seen because most of the circulating fibrin is cleared by the reticuloendothelial system.

However in the generalized Shwartzman reaction, as a result of the second intravenous injection of endotoxin, fibrin cannot be cleared sufficiently rapidly and excess circulating fibrin is filtered by glomerular capillaries which become occluded. Small vessels in other organs also filter fibrin. Both reactions, local and generalized, are inhibited by anticoagulants[29].

The intrinsic pathway of coagulation has been implicated in the disseminated intravascular coagulation (DIC) reactions described above, since the Hageman factor (factor XII) is activated by endotoxin[76]. Activated Hageman factor leads eventually to the conversion of prothrombin to thrombin. Extrinsic factors are also thought to be involved since leukocytes are needed to produce both the local and generalized Shwartzman reactions. Monocytes release tissue factor (TF), which initiates clotting via the extrinsic pathway[77]. The most likely sites where endotoxin acts to initiate coagulation are summarized in Figure 6 below.

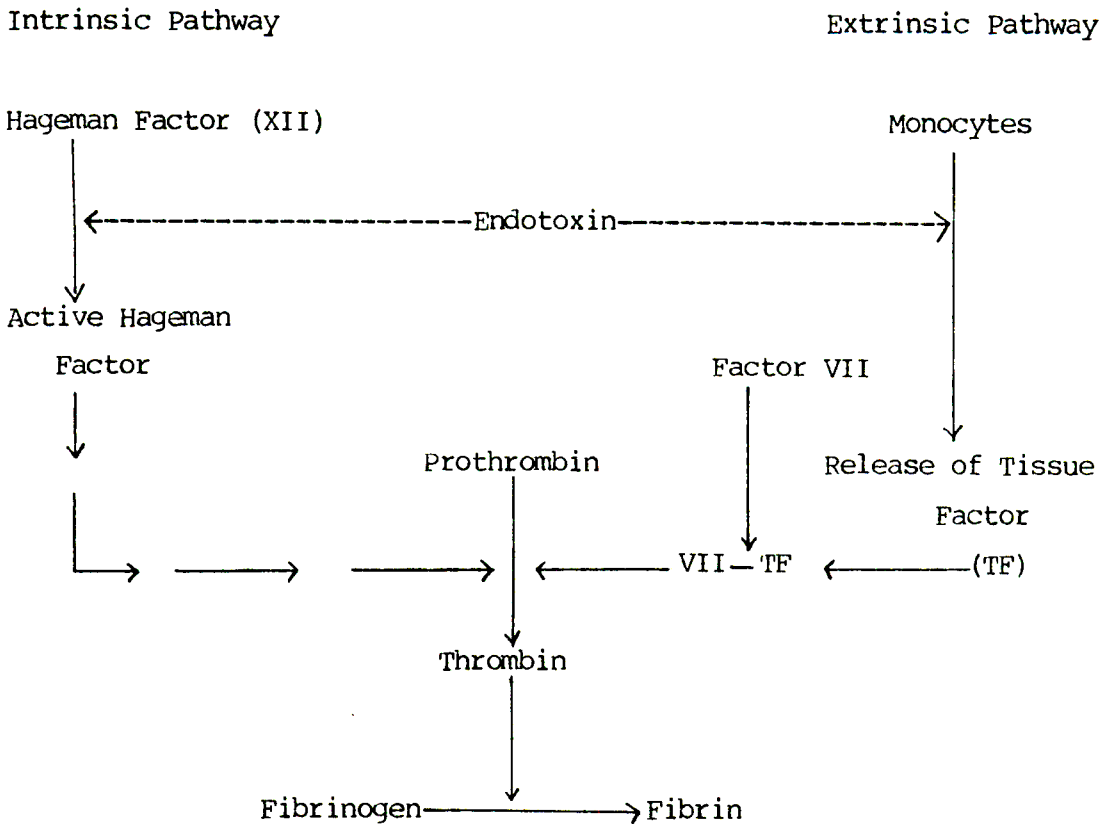


Figure 6. Initiation of coagulation by endotoxin

Activated Hageman factor, in addition to its effect on coagulation, can activate plasma prekallikrein to form kallikrein, a proteolytic enzyme which can generate bradykinin[77]. Active Hageman factor also activates plasminogen.

Disseminated intravascular coagulation eventually leads to a depletion of clotting factors. Moreover, fibrinolysis is also enhanced and fibrin degredation products accumulate in the blood where they act as anticoagulants. These factors contribute to the serious bleeding seen in patients with meningococcaemia and other Gram-negative infections[29,78].

2.3.4 Metabolic effects

2.3.4.1 Effects on lipid metabolism

Gallin and coworkers report marked increases in the serum concentrations of triglycerides or free fatty acids or both in patients with Gram-negative bacteraemia[79]. Studies on rhesus monkeys show that endotoxin significantly elevated serum triglyceride concentrations, leading to impairment of lipid disposal mechanisms[80]. Levin and coworkers however, report that the mean free fatty acid level in their patients with sepsis was not significantly elevated and the mean serum triglyceride level remained normal, except in the case of 5 patients who had elevated levels[81].

Wardle suggests that lipolysis is enhanced in endotoxaemia resulting in high circulating levels of free fatty acids and often of serum triglycerides[82]. This is due to release of adrenalin and noradrenalin as well as an increase in adenylate cyclase activity in fat cells (as a direct effect of lipid A). Release of ACTH, growth hormone and cortisol also contribute to the lipolytic activity. In addition, lipoprotein lipase activity is also depressed.

2.3.4.2 Effects on carbohydrate metabolism

In muscle, disturbances in carbohydrate metabolism caused by endotoxin include decreased utilization of glucose and ketone bodies, with increased catabolism of branched chain amino acids[82]. The blood glucose and insulin levels appear to depend on the degree of endotoxaemia[83]. When cardiac output is still high, the blood glucose level is elevated and the plasma insulin is three times that found in starvation. However, when cardiac output is decreased and blood lactate is elevated a progressive hypoglycaemia results and the plasma insulin level is as low or lower than in starvation. The hypoglycaemia is due to depleted glycogen stores, depressed

gluconeogenesis, both by liver and kidneys (the synthesis of the regulatory enzyme phosphoenolpyruvate carboxykinase is suppressed) and increased tissue utilization of glucose [82,84-86]. The low insulin level is due to adrenalin secretion which, in turn, inhibits insulin secretion[82,83].

2.3.4.3 Effect on serum iron concentration

Hypoferraemia occurs in mice and rats after injection of small doses (0.1 ug) of endotoxin[29]. The fall in serum iron concentration is so reproducible that it has been suggested as a bioassay for endotoxin. An infusion of 5 ng/kg body weight of Lipexal, a highly purified S.typhosa endotoxin in human volunteers resulted in a significant decrease in the concentration of serum iron[70].

2.3.4.4 Other metabolic effects

(a) Endotoxin depresses cellular respiration as a result of impaired mitochondrial functioning[86,87].

(b) Endotoxin causes damage to cell surface phospholipids, for example in the lung alveoli[82].

(c) Endotoxin causes cholestasis[88,89].

(d) Phosporus metabolism is disturbed in Gram-negative septicaemia[90]. In a study on 54 patients with Gram-negative septicaemia, hypophosphataemia is reported in 69% of the cases.

2.3.5 Tumour necrosis

Endotoxin causes haemorrhagic necrosis of tumours in guinea pigs, rats, mice and man[29,91,92].

2.3.6 Abortion

Injection of sublethal doses of endotoxin to pregnant rats and mice resulted in abortion and resorption of the foetuses[93]. Placental haemorrhaging occurs and has been related to serotonin release.

2.4 GRAM-NEGATIVE BACTERIAL INFECTIONS IN MAN

Infections caused by Gram-negative bacteria have become increasingly common. In a study at the University of Minnesota it was found that the number of patients with Gram-negative bacillary bacteraemia has increased from 4,9 per 1 000 hospital admissions in 1958 to 8,1 in 1966[94]. Moreover, the mortality rate due to bacteraemia increased from 37% to 57% in the same period. A study in Boston revealed an incidence of 10,7 episodes of Gram-negative bacillary bacteraemia per 1 000 hospital admissions between 1967 and 1969[95]. The annual frequency, in the United States, of Gram-negative bacteraemia has been estimated at approximately 1 per 100 hospital patients, with fatality rates of 30-50%[5].

Escherichia coli is the species most frequently isolated from blood cultures[94,95]. The Minnesota study points to an increase with time in bacterial isolates of the klebsiella-enterobacter-serratia group[94]. In 1958, only 8 isolates of this group were recorded compared to 25 in 1966. Bacteraemia due to pseudomonas species has shown a less marked increase in yearly isolation.

A similar trend emerged from the Boston study[95]. Table 2 identifies the specific Gram-negative organisms responsible for the bacteraemia.

Table 2

A study of 149 consecutive clinical episodes of bacillaemia due to Gram-negative organisms, during 1967-1969 at the Peter Bent Brigham Hospital, Boston[95].

Gram-negative Species	No. of Isolates	Percentage
Escherichia coli	58	36
Serratia	27	17
Klebsiella	23	14
Pseudomonas	21	13
Proteus	13	8
Enterobacter	9	6
Salmonella	5	3
Bacteroides	2	1
Providence	1	< 1
Unclassified	2	1

Table 3 shows the incidence of the more common Gram-negative bacterial species in blood cultures of patients at the King Edward VIII Hospital in Durban for 1980 and 1981.

Table 3

Common Gram-negative isolates from the blood cultures of patients at the King Edward VIII Hospital in Durban, 1980 and 1981.

Gram-negative species isolated from blood cultures	1980		1981	
	No.	% Total Gram-negative isolates	No.	% Total Gram-negative isolates
Salmonella typhi	429	26	245	18
Klebsiella	285	17	318	23
Escherichia coli	265	16	315	23
Enterobacter (including Serratia)	195	12	63	5
Salmonella sp.	127	8	92	7
Pseudomonas	82	5	102	7

Figures for 1980 were obtained from the Department of Microbiology at the King Edward VIII Hospital (Y.M.Coovadia, pers.comm.*)
Figures for 1981 were compiled from the data of the Antibiotic Study Group of South Africa[96].

The increased and widespread use of antibiotics has been implicated as a major contributing factor to the more frequent apprearance of Gram-negative bacteraemia[97]. Wolff and Bennett comment that the "use of antimicrobial agents tends to promote emergence of gram-negative organisms with intrinsic or acquired resistance to these agents, and predisposes the patient to colonization with resistant exogenous gram-negative organisms"[98]. Serratia marcescens, for example, is resistant to all antibiotics except gentamicin and has become a serious problem in hospital-acquired infections[97].

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A study of neonatal sepsis at the Yale-New Haven Hospital in the United States, revealed that cases due to Pseudomonas aeruginosa first appeared after 1943 and those due to Klebsiella-Enterobacter did not occur until after 1957[97]. Sulfonamides had become widely used by 1943 and by 1951 penicillin, streptomycin as well as the so-called "broad-spectrum" antibiotics (e.g. chlorotetracycline and chloramphenicol) were extensively used.

Other factors also contribute to the increased number of infections reported. Therapeutic agents such as cytotoxic immunosuppressive drugs and adrenocorticosteroids lower host defences and thus enhance susceptibility to infections. Septicaemia is said to be the major cause of death in patients with cancer[4]. In a study of septicaemia in children with cancer the most frequently isolated Gram-negative organisms were reported to be E.coli, Klebsiella pneumoniae and Pseudomonas aeruginosa.

Patient resistance to infection is also lowered by neutropaenia and the immune disorders of leukaemia and lymphoma[3]. It has been claimed that, despite the best available treatment, many leukaemic patients die from pseudomonas and klebsiella septicaemia. Braude suggests that failure of therapy in Gram-negative bacteraemia is due to multiple drug resistance, as well as to the toxicity of the lipopolysaccharide[3].

Patients with neoplasia, cirrhosis, diabetes mellitus and other conditions are surviving longer than in the past[94,98]. Such patients, whose defence mechanisms are often compromised, are more susceptible to bacterial infections. Gram-negative bacteraemia is thought to be more prevalent in older persons and the greater proportion of elderly persons among hospitalized patients is yet another factor contributing to the increase in Gram-negative bacterial infections[98].

Finally, there is a marked increase in the number of surgical procedures being carried out[94]. These include, for example, manipulations of the genitourinary and gastrointestinal tracts, which are, in turn, important sites for the entry of bacteria into the circulation. Similarly, the more frequent use of devices such as intravenous and bladder catheters and inhalation equipment is also implicated in the spread of bacterial infections[98].

2.5 CONVENTIONAL THERAPY FOR ENDOTOXAEMIA

2.5.1 Introduction

Endotoxaemia can now be identified using the Limulus amebocyte lysate technique [81]. If the endotoxaemia is derived from a septic focus, this must be removed[99]. In the absence of a septic focus, the intestines have been shown to be a major source of endotoxin. In such cases there may be a failure of the reticuloendothelial system in the liver to clear and destroy endotoxin entering the circulation.

Fine and colleagues suggest that "treatment should be primarily concerned with eliminating the endotoxaemia and preserving what is left of the antibacterial potential in the R.E.S."[99]. They consider that "the main thrust of therapy will be wrongly directed if it is aimed, for example, at the respiratory disability which is secondary to an endotoxaemia".

Nevertheless, the basic principles guiding therapy in septic shock are volume expansion, correction of metabolic abnormalities, for example restoration of normal blood pH, and correction of hypoxia by providing respiratory assistance.

Plasma expanders are used in an attempt to restore intravascular volume[100]. The best choice of fluid type remains controversial. If the haematocrit is low, whole blood or packed red cells are used. For

other patients a choice is made between colloidal (e.g. low molecular weight dextran, albumin) and crystalloid solutions. However, patients in endotoxin shock may not respond well to volume replacement. In endotoxaemia, microcirculation abnormalities result in "leaky" capillaries so that administered fluid is transferred into the interstitial fluid resulting in oedema. Seventy-five percent of the administered electrolyte solutions leave the vascular space within minutes and may lead to congestive heart failure[100]. Fine and coworkers using a haemorrhagic shock model, report that "large volume intravenous infusion therapy, using either physiologic saline solution alone or albumin in physiologic saline solution, is harmful by producing marked edema of tissues, pulmonary edema, serous effusions, venous distension and widespread hemorrhage from small vessels"[101].

Vasoconstrictors have been used in an attempt to reverse the hypotension caused by endotoxin[102]. However, these agents improve cardiac output by diverting blood from the vascular beds of the kidney, gut, liver and extremities. The generally accepted principle that intense vasoconstriction is a self-defeating mechanism has led to investigation of anti-adrenergic therapy in experimental shock. Drugs such as phenoxybenzamine have been found to be effective when used prophylactically but are useful in preventing death from endotoxic shock in experimental animals only if administered within thirty minutes after onset of the shock state[103].

The use of various drugs and antibiotics in the treatment of septic shock is outlined below. Other treatments, such as extracorporeal haemoperfusion, are also examined.

2.5.2 Steroids

The use of large but not small doses of corticosteroids for treatment of septic shock and endotoxaemia have been shown to be beneficial both in animal models and clinically[104-107]. Corticosteroids have been

shown to increase survival time particularly when given prophylactically[105].

Endotoxin-induced hypoglycaemia and lacticacidaemia were alleviated by administration of glucocorticoids[108].

Glucocorticoids have been shown to prevent the disseminated intravascular coagulation induced by endotoxin[109]. In rats, glucocorticoids interfere with the in vivo mechanism of activation of Hageman factor as well as with the availability of platelet procoagulant activity. As a consequence, vasoactive substances such as serotonin, and the kinins are not released.

Steroids are able to stabilize lysosomal membranes and have been shown to prevent the release of hydrolytic enzymes into the circulation, during shock[110]. O'Flaherty and colleagues suggest that certain complement-inhibiting effects of corticosteroids, particularly their ability to inhibit formation of a neutropaenia-inducing substance (NIS), may underlie their protective role in endotoxaemia[111].

The ability of corticosteroids to increase survival time in septic shock may be related to protection against a haemoconcentration[105]. This could be the result of partial protection against the increased vascular permeability seen in septic shock.

Fine and colleagues report that corticosteroid treatment prevents the endotoxaemia of intestinal origin which results when rabbits are subjected to immersion burns[104].

Steroids are thought to prevent the excessive release of noradrenalin by endotoxin[104].

2.5.3 Indomethacin

Prostaglandins have been reported to be released following LPS administration in man and in experimental animals[42]. Drugs which block prostaglandin synthesis or release, such as indomethacin have been shown to influence the course of the shock state.

Parratt and Sturgess have demonstrated that in cats, indomethacin administration prior to E.coli endotoxin injection prevented the initial pulmonary vasoconstriction and alleviated some of the characteristic features of delayed endotoxic shock[112]. However, when they studied the effects of indomethacin administration 30 minutes after injection of E.coli endotoxin, the cats all exhibited the characteristic features of the shock phase, i.e. hypotension, hypoglycaemia, reduction in cardiac output and a marked metabolic acidosis[113].

Multiple doses of indomethacin were found to protect dogs against E.coli endotoxin-induced shock[114]. Plasma volume losses of indomethacin treated animals were substantially less than in untreated animals. In this study, nicotinic acid was found to have a similar effect to that of indomethacin. It is postulated that nicotinic acid prevents the release of fatty acid precursors of prostaglandins and consequently reduces formation of prostaglandins. Indomethacin has also been shown to improve the survival of baboons in endotoxic shock[115].

Fletcher and Ramwell have compared the effectiveness of indomethacin and aspirin treatment of endotoxaemia in dogs and find that although all the dogs in both treated groups survive, only those treated with indomethacin show improved haemodynamic status after endotoxin injection[116]. Both indomethacin and aspirin were equally effective inhibitors of the increase in circulating prostaglandin evoked by

endotoxin. It is suggested that the early haemodynamic events in the dog may not necessarily be related to survival since aspirin treatment did not alter these events and that the primary factor in survival in the dog may not be inhibition of prostaglandin synthesis but may be related to other actions of aspirin and indomethacin.

In contrast to the studies described above, it is noteworthy that there is also some evidence to show that administration of components of the arachidonic acid-prostaglandin system will improve the shock state[117]. There are reports of improvement in blood pressure and cardiac output during endotoxin-induced shock with PGE_1 and PGA_1 infusions; however, there is no improvement in survival.

Fletcher and Ramwell have investigated the effects of prostacyclin (PGI_2) treatment in a canine endotoxin model[117,118]. PGI_2 is a major metabolite of arachidonic acid and is synthesized in vascular endothelial cells. It is a potent inhibitor of platelet aggregation and a vasodilator. Survival was increased to 83% over the control level of 42%. The authors suggest that the synthesis and release of PGI_2 may be inhibited during shock, thereby allowing the vascular endothelium-platelet interaction to be altered, in which case platelets and other formed elements would adhere to vessel walls and impede microcirculatory flow.

Although there may be some therapeutic benefit in the administration of components of the arachidonic acid-prostaglandin system, it is more likely that drugs used to inhibit the prostaglandin synthesis and/or release are more effective in improving survival[117].

2.5.4 Lidocaine

Lidocaine in therapeutic doses has been shown to improve the survival of baboons in endotoxic shock[115]. The mechanism by which lidocaine improves survival is not known, however it is known that lidocaine

stabilizes cell membranes and decreases membrane permeability. It is also known to decrease mesenteric vasoconstriction during endotoxin shock.

2.5.5 Dopamine

Dopamine is known to cause vasodilatation in the renal vascular bed[119]. Infusion of dopamine in baboons receiving endotoxin improved the cardiovascular status of the animals. Dopamine infusion improved the blood flow to the kidney which was attenuated by endotoxin.

2.5.6 Naloxone

Endogenous opiates are thought to be released due to the action of endotoxins and may contribute to reduction of blood pressure. Naloxone, a specific opiate-antagonist, was found to temporarily reverse endotoxin-induced hypotension in experimental animals[61,62].

2.5.7 Proteinase inhibitors

Endotoxins damage the membranes of leucocytes and other cells causing certain cell components including lysosomal enzymes to be released. The action of these enzymes include the non-specific proteolysis of blood proteins[52].

The proteolytic activity of elastase, cathepsin G and other proteinases from polymorphonuclear leucocytes is inhibited *in vivo* by complex formation with endogenous inhibitors. During septic shock, consumption of proteinase inhibitors is evident and the endogenous inhibitor system of the organism may be overstressed due to a massive release of lysosomal proteinases.

The elastase-cathepsin G inhibitor from soya beans and a broad

spectrum proteinase inhibitor aprotinin have been used in the treatment of endotoxaemia in dogs. With the simultaneous administration of endotoxin and inhibitor the consumption of plasma factors corresponded to that of control animals. The degradation of Factor XIII, a sensitive substrate for granulocytic elastase, was almost completely prevented by elastase inhibitor.

While animal studies appeared promising, no successful clinical trials on humans have been reported.

2.5.8 Antibiotics

The value of antibiotics in the treatment of Gram-negative sepsis is limited. Many bacteria have become resistant to various antibiotics and their sensitivity to a specific drug can no longer be simply deduced from species identification[120]. *In vitro* measurement of drug susceptibility is required to measure the ability of a drug to inhibit growth of a pathogenic organism.

There has, however, been considerable interest in the polymyxins. Polymyxin B, a cyclic polypeptide, has been shown to have unique antiendotoxin properties, protecting against injury by lipopolysaccharide by disrupting its structure as shown by electron microscopy[121]. Polymyxin B has been shown to neutralize endotoxin effects in animals given purified endotoxin, dead endotoxin-containing organisms and in Gram-negative septicaemic animals[122]. The antiendotoxin effect of the antibiotic appears to be separate from its antimicrobial effects.

Although the exact mechanism of the protective effect of polymyxin B against endotoxin is unknown there appears to be interaction between the cationic antibiotic and the negatively charged KDO - lipid A moiety of the endotoxin[123]. In the intact bacterial cell, this interaction results in complete loss of structural integrity of the

outer membrane.

Bannatyne and coworkers have found that polymyxin B diminishes the endotoxin mediated release of human blood neutrophil lysosomal enzymes and suggest that, in addition to having endotoxin inactivating properties, polymyxin B may also act by blocking access of endotoxin to surface receptor sites on the neutrophil[124].

Polymyxin B has been used clinically in the treatment of endotoxaemia, for example, in patients with cirrhosis[125,126]. The usefulness of polymyxin B is however limited since it is nephro-toxic[127]. The low doses administered do not give blood levels adequate for severe systemic infections[120].

Table 4 below lists common Gram-negative organisms and some of the antibiotics used in their treatment[120]. Some of these antibiotics are highly toxic, for example, gentamicin and kanamycin, while others like carbenicillin, a semi-synthetic penicillin, have a tendency to produce drug resistance.

Table 4

Common Gram-negative organisms and preferred antibiotics[120]

Organism	Drugs
E.coli	ampicillin, cephalothin, kanamycin gentamicin
Klebsiella	cephalothin, kanamycin
Enterobacter	kanamycin, carbenicillin, gentamicin
P.mirabilis	ampicillin, cephalothin
Indole-positive Proteus	kanamycin, carbenicillin, gentamicin
Pseudomonas	carbenicillin, gentamicin, polymyxin B or E.

2.5.9 Fibronectin

Humoral opsonic factors modulate the phagocytic behaviour of the cells of the reticuloendothelial system[128]. One such opsonic factor is a circulating protein fibronectin also called opsonic α_2 surface binding glycoprotein or cold-insoluble globulin. An insoluble form of fibronectin is also found in connective tissue, basement membranes and on various cell surfaces. Saba has suggested that "multiple organ failure occurring in patients who develop severe sepsis after trauma, burn or operation may be, in part, related to coexistent phagocytic dysfunction mediated by opsonic fibronectin deficiency". Plasma fibronection deficiency and associated opsonic deficiency in septic shock patients can be reversed by intravenous infusion of fresh plasma cryoprecipitate which is rich in fibronectin. However, the therapeutic value of fibronectin therapy is in dispute.

2.5.10 Glucose therapy

Sepsis due to Gram-negative organisms results in hypoglycaemia due to impaired gluconeogenesis, depleted glycogen stores and an increased rate of glucose oxidation[82,84-86].

Glucose infusions have been shown to have a curative effect in Gram-negative septicaemia. Dogs received an infusion of E.coli and those with elevated blood glucose levels showed a 1000-fold decrease in circulating bacteria within 2 hours when compared with control animals[129]. Hinshaw and coworkers have shown that in experimental endotoxic shock in dogs exogenously administered glucose improves certain cardiovascular and metabolic parameters and prevents death[130].

2.5.11 Extracorporeal haemoperfusion

The conventional treatments used for endotoxin shock do not usually

have a detoxifying effect on the circulating levels of endotoxin. It has therefore been suggested that some method of diluting or removing the circulating endotoxin be employed.

The use of haemodialysis to remove endotoxin from the circulation is precluded since the molecular size of endotoxin is too large to be dialyzed by currently available haemodialysis units[131].

Nakamura and coworkers injected rabbits with E.coli endotoxin and two hours later treated them with a total body washout (TBW) i.e. total removal of endotoxin-contaminated blood followed by its replacement with fresh blood[131]. Although this did improve survival in rabbits, the drastic procedure is unlikely to be used clinically as a general technique. Total body washout has been employed in a few human cases; Klebanoff and coworkers report the use of the technique in treatment of stage IV hepatic coma[132].

Endotoxin binds nonspecifically to charcoal and it has been removed from human blood by perfusing the blood through an extracorporeal charcoal filter[133]. Sterile columns of purified activated charcoal which have been coated with an acrylic hydrogel are used for this purpose. The gel reduces adsorption of platelets which would otherwise lead to severe thrombocytopaenia. Gazzard and coworkers treated 22 patients in grade IV coma from fulminant hepatic failure with charcoal haemoperfusion and found that the procedure was well tolerated with 11 of the patients regaining consciousness and ten leaving hospital. However, in a subsequent study, of 34 patients treated, only three survived, and many had severe hypotensive reactions[134].

2.5.12 Summary

Some of the treatments used in an attempt to lower the high mortality rate from Gram-negative bacteraemia have been described. However, despite the use of powerful antibiotics, high doses of adrenocorticosteroids, modern life-support systems and sophisticated monitoring devices, the incidence of bacteraemia continues to increase, with sustained high mortality rates[6]. An important new approach to therapy for Gram-negative bacteraemia has been the introduction of human antibodies directed against endotoxins. The studies which have led to the development of these antiendotoxin antibodies are summarized in section 2.6.7 (Antiendotoxin antibodies). Clinical trials, which have shown the effectiveness of immunotherapy for Gram-negative bacteraemia, are also discussed.

2.6 HOST DEFENCE SYSTEMS AGAINST ENDOTOXINS

2.6.1 Introduction

Normal host defence against microbial invaders depends on an intact humoral and cellular system of inflammation and specific immunity. It has been aptly said that "these (endotoxins) molecules are read by our tissues as the very worst of bad news. When we sense lipopolysaccharide, we are likely to turn on every defense at our disposal"[135]. The major mechanism for killing bacteria is the action of phagocytic cells. However, humoral factors enable these cells to recognize, migrate towards and, ultimately, kill the invaders. Humoral factors include antibacterial immunoglobulins plus the proteins which comprise the complement system.

Some of the principal effects of endotoxins on host cellular mediation systems are outlined below. The effects of endotoxin on the complement system and on antibody formation are then discussed.

2.6.2 Polymorphonuclear leukocytes - granulocytes

Endotoxin-induced leukocytosis (i.e. increase in the number of leukocytes) occurs in humans at subpyrogenic doses (i.e. $<10^{-9}$ g/ml) and is secondary to the release of granulocytes from the granulocyte reserves[70,136]. The neutropaenia (reduction in neutrophil concentration in the blood) common to other animals is however not always seen in man. Higher doses of endotoxin are required for neutropaenia than for leukocytosis[70]. Neutropaenia results from the sequestration of neutrophils by capillaries of the lung and other organs[136]. Corrigan and coworkers suggest that the lipid A portion of endotoxin is critical in the induction of neutropaenia following endotoxin injection[137].

(The role of complement proteins in the mediation of the phagocytic activity of granulocytes is discussed in the section on complement, 2.6.6).

2.6.3 Mononuclear phagocytes - monocytes and macrophages

The blood monocytes and tissue macrophages are important in host defence against microbial invaders not only as a result of their phagocytic properties but also because they may be stimulated to release various substances such as prostaglandins and collagenases[77]. These cells are particularly sensitive to the presence of endotoxins, many responses being observed in vitro with the addition of submicrogram quantities of endotoxin.

Endotoxins activate monocytes, enhancing their ability to phagocytose bacteria[138]. Monocytes undergo marked changes in both shape and cytoplasmic organization in response to endotoxin. Endotoxins also stimulate the formation of lysosomal hydrolases by monocytes.

Wahl and coworkers report that activation of peritoneal macrophages by endotoxin, particularly the lipid A component, induces the cells to produce collagenase[139]. The production of collagenase by the endotoxin-stimulated macrophages was significantly inhibited by indomethacin, suggesting that prostaglandins mediate this effect[140].

Exposure to endotoxin enhances the production of plasminogen activator (a trypsin-like serine protease) by macrophages[141].

Endotoxins can stimulate peritoneal exudate cells to become cytolytic for tumour cells[142].

Exposure of murine blood monocytes, spleen cells or peritoneal exudate cells to endotoxin results in secretion of a colony stimulating factor (CFS) which is required for the growth of granulocytic and monocytic cells *in vitro*[77].

Purified populations of both human peripheral blood monocytes and murine peritoneal macrophages have been shown to synthesize and release prostaglandin E *in vitro*[143]. This production of prostaglandin E by macrophages is markedly enhanced by endotoxin and completely suppressed by indomethacin.

2.6.4 Lymphocytes

Endotoxin stimulates proliferation of a large subpopulation (nearly 50% of spleen cells of murine strains studied) of lymphoreticular cells[144]. This mitogenic effect is elicited by the lipid A component of endotoxin and was shown to be thymus cell-independent and a property of a low-density cell subpopulation, probably bone marrow-derived (B) lymphocytes.

2.6.5 Platelets

Endotoxin-induced thrombocytopaenia is seen in certain experimental animals, for example, rabbits, guinea pigs and dogs[77]. Primates, including man, are much less susceptible to thrombocytopaenia after endotoxin injection than are lower mammals. Only small decreases in platelet counts are reported in studies on human volunteers receiving two endotoxin injections[70].

One of the mechanisms of the platelet-endotoxin interaction depends on the presence or absence of immune adherence receptor sites on the platelet membrane[77]. (The aggregation of blood cells in the presence of antigen, antibody and the first four components of complement has been termed immune adherence. Receptors mediating immune adherence are present on platelets in non primate species, but in primates the erythrocyte is the reactive cell[145]). Platelets from primates do not respond significantly to endotoxins since they lack immune adherence receptor sites.

Platelet responses are characterized by aggregation and the release of platelet constituents, for example, ADP, platelet factor 3, histamine and serotonin[77].

2.6.6 COMPLEMENT

The complement system present in plasma consists of a series of inactive proteins which may be activated in a sequential manner[146]. Active complement components help to bring about the destruction of bacteria. The complement system can be activated in at least two different ways. The so-called "classical" activation sequence involves antibodies (IgG, IgM). These antibodies activate the first complement component (C1 which then activates the second and fourth

components (C2 and C4). This leads to activation of the third component (C3). Thereafter, a terminal sequential cascade of reactions is triggered, activating components C5 to C9.

The "alternative" pathway can be activated, without involving antibodies, by various substances including polysaccharides such as inulin, zymosan and endotoxin[147]. These components also trigger activation of C3 leading to the same terminal cascade of enzymatic reactions as in the classical pathway. The two pathways are summarized below in Figure 7.

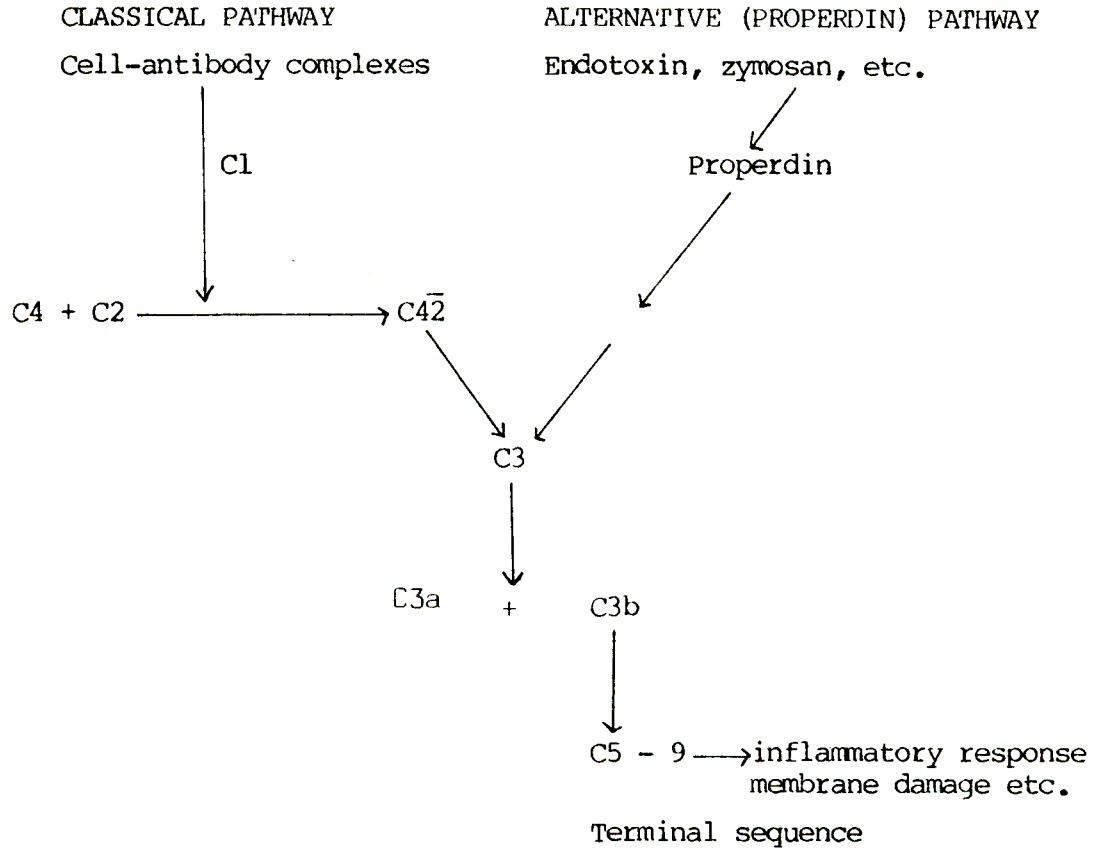

Figure 7.An abbreviated scheme of the two pathways of complement activation

Membranes are the primary target of complement[146]. They may be irreversibly damaged, sustaining distinct ultrastructural lesions. In general, Gram-negative organisms are susceptible to the action of complement, while Gram-positive species are resistant. The differential susceptibility has been linked to differences in phos pholipid content and thickness of the cell wall.

An important role of the complement system is to potentiate the capability of granulocytes to kill bacteria[148]. C3b, a cleavage product that results from activation of C3 is able to opsonize bacteria i.e. alter bacteria so that they are more readily and efficiently engulfed by phagocytes. Granulocytes and monocytes have receptors for this and other complement components and so readily attach to and ultimately phagocytose complement coated bacteria. Granulocyte migration to the sites of infection is also directed by complement components. Thus, the complement system not only prepares bacteria for phagocytosis by coating them with C3b but it also produces substances that attract phagocytic cells to the bacteria. Individuals who are genetically deficient in various complement components suffer chronically from infections[149].

Several of the in vitro biological activities attributed to components of complement or products of their cleavage are significant in the pathophysiology of the endotoxin reaction[150]. Some of these activities are summarized in table 5.

Table 5

Biological activities produced *in vitro* as a consequence of interactions between endotoxin and complement[150].

Biological activity	Complement components involved
Anaphylatoxin	C3, C5
Contraction of smooth muscle	
Increased capillary permeability	
Release of histamine and heparin from mast cells	C3, C5
Adherence and degranulation of platelets	C3
Coagulation of blood	C6
Chemotactic factors	C5
Polymorphonuclear leukocytes	
Mononuclear leukocytes	

Experiments of Spink and Vick suggest that complement components may be involved in the lethal effects of endotoxins in dogs[151]. Dogs were protected against the lethal effects of endotoxin by transfusions of blood, in which an "essential serum factor" (possibly complement components) had been depleted by heating plasma at 56°C for 30 minutes, prior to administration of endotoxin.

The action of complement is not always beneficial[77,149]. The reactions mediated by the system are not always contained and modulated. In many cases of Gram-negative bacteraemia, there appears to be an overactivation of the complement system. It is suggested that high concentrations of bacteria in the circulation provide a continuous stimulus to activation and amplification of complement proteins. This could help explain why there is often a depletion of complement in the serum of bacteraemic patients. As a result, the inflammatory response can become exaggerated, producing increased

vascular permeability, leakage of blood into the interstitial space and release of lysosomes from white cells. Thus in many cases, the host response to the Gram-negative organism, rather than the organism itself, poses the ultimate threat to host tissues.

There has been considerable interest in the effect of endotoxin on complement components. The results of some of the in vivo studies are summarized below.

Landy and Pillemer report that injection of endotoxin into mice induced increased levels of circulating properdin (a major protein component of the alternative pathway)[152,153]. The properdin titre was elevated to levels two to three times the normal. The rise in properdin levels is correlated to an increase in resistance to certain bacterial infections. Following infection with Gram-negative organisms, in control animals, the properdin titres progressively declined and the animals died, while in mice pretreated with endotoxin, properdin levels were maintained in the normal range or increased.

In contrast Hook and coworkers report a drop in properdin levels following injection of S.typhosa endotoxin into mice[154]. Gilbert and Braude report that injection into rabbits of a lethal dose of endotoxin produced a rapid and sustained fall in complement levels, however, doses below LD_{50} caused only sporadic alterations of complement titres[155]. In a similar study it was found that serum complement activity declined even after injection of adjuvant amounts of endotoxin into rabbits[156].

McCabe has studied alterations in complement levels in patients with bacteraemia due to Gram-negative organisms[157]. In patients with uncomplicated bacteraemia the mean C3 levels were not found to differ from values in controls, however, a significant reduction in C3 levels were observed in patients with shock and in those who died. Fearon

and coworkers provide evidence of activation of the alternative, properdin pathway and the terminal complement sequence in patients with bacteraemia resulting in shock[158].

On the other hand, a recent study reveals no significant differences in complement values between patients with non-septic shock and healthy individuals[159]. Gram-positive septic shock showed a pattern of complement activation similar to that of Gram-negative septic shock and was characterized by a fall in whole serum complement, C3 and C4, indicating classical pathway activation.

The role of endotoxin-initiated activation of complement on endotoxin activities in vivo, has also been examined. It has been suggested that C3 may cause some of the pathological lesions induced by endotoxins[160]. Anti-complement serum (which is directed mainly against the third component) suppressed the appearance of haemorrhage in guinea pigs. Consistent with these results are those of Fong and Good who depleted rabbits of C3 and the terminal complement components and then induced localized and generalized Shwartzman reactions[161]. A cobra venom anticomplementary factor was used to consume C3 and terminal components. Only one of the fifteen complement-depleted rabbits developed either the local or generalized Shwartzman reaction while most of the control animals developed lesions.

In contrast, other workers using the same experimental system, report completely opposite results. Bergstein and Michael were unable to prevent the development of Shwartzman reaction lesions by prior depletion of C3 and terminal components[162]. The sixth component of complement as well as components C7-9 were also not found to be essential to the initiation of intravascular coagulation by endotoxin[163]. Similarly, prior depletion of complement components in rabbits failed to alter the hypotension induced by injection of endotoxin[164].

Brown and Latchmann, however, show that prior C3-9 depletion appears to be protective and correlates well with absence of mortality, normal platelet survival and failure to activate platelet factor 3 (PF3)[165]. Similarly, injection of endotoxin into rabbits genetically deficient in C6 resulted in a high death rate, whereas rabbits with a partial deficiency of C6 as well as rabbits with normal levels of C6 survived[166]. Thus, a critical role for C6 is suggested in the protection of hosts against the lethal effects of endotoxins.

In an attempt to reconcile some of these conflicting results Morrison and Ulevitch suggest that "differences in endotoxins from different species and from the same organism prepared by different procedures, as well as inherent differences in these various endotoxins to activate the various complement pathways, will contribute to their capacity to affect the complement system in vivo"[77]. They conclude that "the interaction of complement with bacterial endotoxins in vivo does, at least in part, contribute to the overall host response to endotoxin".

Endotoxins are thought to activate both the classic and the alternative complement pathways. Endotoxin was injected into normal guinea pigs and into guinea pigs congenitally deficient in the fourth component of complement (C4)[167]. The C4 deficient guinea pigs are known to have a complete block in the activation of the classical complement pathway, but with the alternative pathway intact. In both sets of guinea pigs there were significant drops in the levels of C3-9 after endotoxin injection.

It appears that in the classical mechanism of complement activation by endotoxin, the antiendotoxin antibody-endotoxin complex activates C3. In addition, lipid A binds directly to C1 leading to a "classic" pathway which is not antibody dependent[77]. In the alternate pathway antibody is not involved at all.

2.6.7 Antiendotoxin antibodies

Investigators in the 1940's established that increased resistance to endotoxin toxicity could be generated by vaccination of animals or individuals with endotoxin or killed Gram-negative bacteria.

Favorite and Morgan found that in humans, a purified typhoid pyrogen induced a fever of from 103 to 105°F when administered intravenously in doses of from 0,001 to 0,002 mg.[7]. However, in order to obtain similar results with subsequent injections, the amount of pyrogen had to be increased by three- to fivefold.

Rabbits are shown to develop tolerance to the pyrogenic action of endotoxins from S.typhosa, S.schottmuelleri or Sh.dysenteriae following repeated intravenous injections[8]. Animals that have been injected with any of these three antigens showed a diminished febrile response following injection of either of the two heterologous antigens. Morgan believed this acquired resistance to endotoxin to be independent of antibody levels in the circulation. A similar view was held by Beeson who failed to accomplish the passive transfer of the tolerance[168]. Sera from rabbits who had been injected with a S.typhosa vaccine were pooled and administered intravenously to rabbits in doses of 10 ml prior to the animals being given the typhoid vaccine. Control animals received normal rabbit serum. The febrile response of all the rabbits, experimental and control, fell within the range of ordinary responses and there was no appreciable difference in the fevers in the two groups.

On the basis of other studies, Beeson suggested that tolerance to pyrogens develops as a result of a change in the functional capacity of the reticulo-endothelial system whereby the ability to remove bacterial toxins from the blood is enhanced[169].

In 1959, Freedman reported that the passive transfer of serum from endotoxin-tolerant rabbits protected mice against homologous and heterologous endotoxins[9]. The "tolerant" serum lost its activity on heating. Freedman does not however attribute the protective effect to the antibody content of the serum. He found that the serum of endotoxin-tolerant rabbits contained low or no antibody titres to the endotoxins and suggests that "low antibody titers to endotoxin would be expected from the weak antigenicity of lipopolysaccharides"[9]. Abernathy and Spink found that sera from patients with brucellosis contained "precipitins" for brucella endotoxin and conferred passive protection against the lethality of brucella endotoxin for mice[10]. The sera from 9/19 patients with brucellosis protected mice against the lethality of brucella endotoxin. None of the control sera protected mice. It was also found that injections of brucella endotoxin into humans caused the formation of serum "precipitins" for the endotoxin.

Ritts and coworkers found that tolerance to a specific endotoxin was the result of a specific rise in antibody with only a slight-to-moderate increase in heterologous antibody[170]. They showed that "tolerant" serum, containing high concentration of specific antibody, when transferred to normal mice gave absolute protection against a lethal combination of bacteria and endotoxin. When this serum was heated for 1 hour at 56^{o}C and transferred to normal mice, the mortality rose to 83%.

By 1964 Landy and Weidanz concluded that "it should be evident that in experiments in animals and man which involve parenteral injection of endotoxin, intravascular antigen-antibody reactions are produced"[171].

In order to investigate the immune responses to infection, Braude and his coworkers injected E.coli 0:113 into the knees of rabbits and established a severe arthritis which was accompanied by fever lasting

two weeks[172]. Endotoxin but not bacteria was found in the blood of these febrile animals. Antibody titres against E.coli endotoxin appeared at their maximum level on about the seventh day after infection with E.coli. From this time on there was a decline in the fever peaks, even though endotoxin was shown to persist in the infected knees long after. It is suggested that antibodies might neutralize the pyrogenic properties of endotoxin persisting in the knee. To examine this further, rabbit antiserum with a high titre of antibodies was prepared by repeated intravenous injections of endotoxin from E.coli 0:113. Immune serum was injected with 1,0 µg of endotoxin into rabbits and was found to markedly inhibit the pyrogenic response by reducing or abolishing the second peak of the fever curves observed with endotoxin and normal serum. This is illustrated in Figure 8 below.

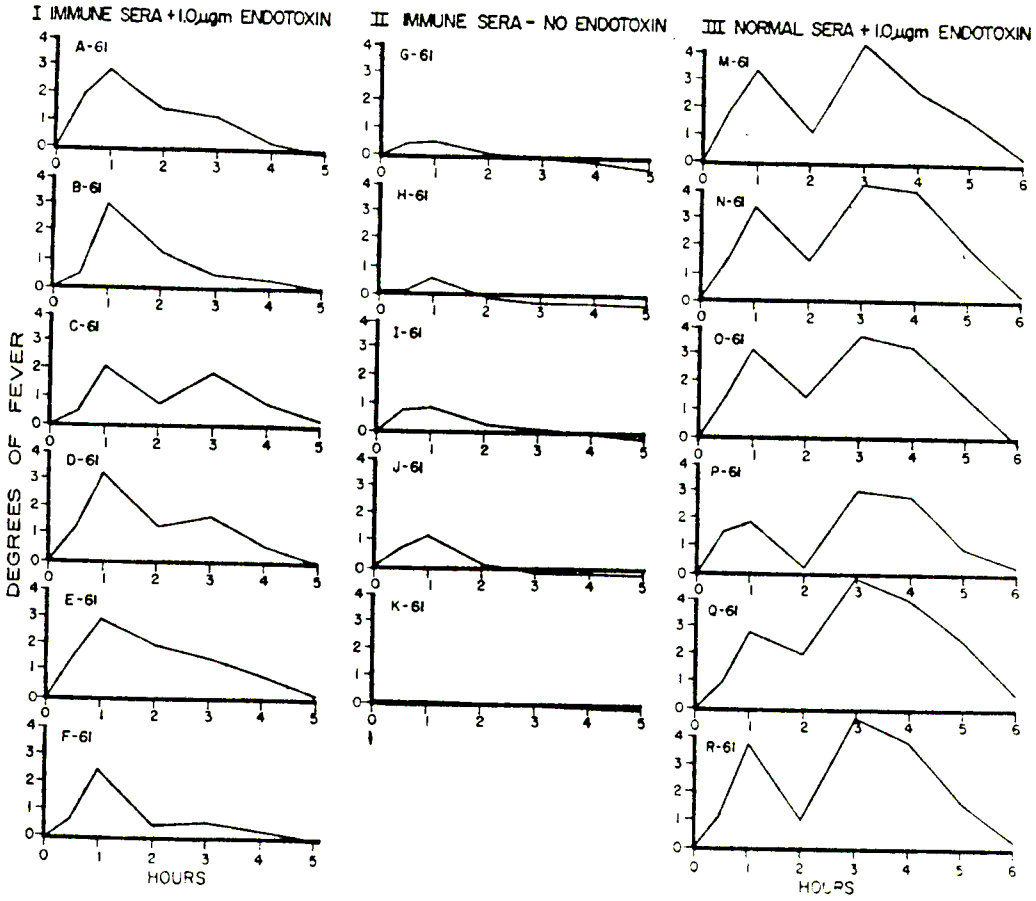

Figure 8. Effects of antibody on pyrogenic response to Escherichia coli endotoxin[172].

E.coli infection was shown to stimulate the production of protective antibodies against endotoxin[173]. Subcutaneous injection of antiserum from rabbits immunized with boiled cells of E.coli O:113 was able to reduce the lethality of E.coli O:113 endotoxin in mice by 50-80%. Antiserum to an E.coli O:113 mutant whose endotoxin was deficient in side chains was also found to be just as protective. Significantly, antiserum against S.abortus equi gave just as much protection against E.coli endotoxin as homologous antiserum. From these observations it was suggested that antibody to the type-specific oligosaccharide "O" side chains of endotoxin, was not necessary for protection. These results were confirmed by a later, extensive study in which it was found that protection was not limited to the homologous antisera but that antisera against endotoxins from unrelated bacterial species also showed a wide range cross-protection[174].

Rabbit antiserum, with high titres of antiendotoxin antibodies was shown to be effective in preventing the local Shwartzman reaction to homologous endotoxin when compared to nonimmune rabbit serum[175]. Antiserum to E.coli O:111 endotoxin reduced the frequency of Shwartzman reactions from 98% to 9,6% in animals given E.coli O:111 endotoxin. Antiserum to S.typhimurium endotoxin reduced the frequency of Shwartzman reactions from 100% to 30,3% in animals given S.typhimurium endotoxin. S.marcescens antiserum cut in half the frequency of the reaction in rabbits given S.marcescens endotoxin. S.typhimurium antisera reduced the frequency of E.coli O:111 induced local Shwartzman reactions in rabbits from 98% to 50%. When the experiment was reversed, E.coli O:111 antiserum did not reduce S.typhimurium induced local Shwartzman reactions. However, antiserum to a epimerase-deficient rough mutant, J5, of E.coli O:111 did prevent S.typhimurium from producing the local Shwartzman reaction. The endotoxin of the J5 mutant is deficient in "O" antigenic units. It was suggested that antiserum prevents the Shwartzman reaction by

acting against a toxic antigen common to endotoxins from unrelated bacterial genera. Loss of the "O" antigenic units was thought to enhance the production of protective antisera, i.e. core antigens, shared in common by E.coli and S.typhimurium are "unmasked" when "O" specific side chains are not synthesized[175].

The ability of antiserum to prevent the local Shwartzman reaction suggested that such serum might also prevent the generalized Shwartzman reaction with its attendant disseminated intravascular coagulation. Rabbit antiserum was shown to prevent intravascular coagulation if given before the first endotoxin injection and to arrest the reaction if given immediately before the second endotoxin injection[176]. Intravascular coagulation was also prevented by antiserum to both heterologous bacteria and side-chain - deficient mutants. The protection by antiserum was found to be independent of complement.

The protective power of the antisera was now investigated in a lethal bacteraemia model. Most of the studies so far described involve the direct intravenous injection of bacteria or their endotoxins into experimental animals. A model was now developed in order to reproduce in animals the form of bacteraemic shock that sometimes follows instrumentation[177]. Bacteraemic shock in man sometimes develops after pelvic instrumentation and invasion of the bloodstream by E.coli and other Gram-negative organism normally resident in the colon. Their method is based on the fact that most of their laboratory rabbits had low concentrations of coliform bacteria in their bowel. Enteric Gram-negative organisms can therefore be introduced selectively into the intestinal tract. Bacteria were fed to coliform-free rabbits in their drinking water and their faeces soon became heavily populated with the organisms. Nitrogen mustard was then given intravenously to produce granulocytopaenia, which is a common feature of human bacteraemia. A temperature probe was inserted into the rectum to mimic pelvic instrumentation of patients as well as

to detect development of fever. The bacilli invaded the pelvic veins; the animals developed high fever, vascular collapse and died. In summary, the model resembles lethal bacteraemia in humans with respect to the endogenous source of the bacteraemia, the immune disturbances and the prominence of vascular collapse.

Ziegler and coworkers used the above technique to produce bacteraemia in rabbits, with either Klebsiella pneumoniae, E.coli 017 or E.coli 04[178]. The animals were treated with antiserum produced by immunization of rabbits with the UDP-galactose-deficient mutant, J5 of E.coli 0:111. When normal serum was given intravenously at the onset of bacteraemia with any of the three organisms, only 3% to 6% of the animals survived. When animals received antiserum to the J5 mutant, the survival rates were 33,3% from E.coli 04, 40% from Klebsiella, and 69,2% from E.coli 017 (a multiple antibiotic-resistant strain). Antiserum to the parent E.coli 0:111 (with the "O" antigens intact) provided no significant protection. Thus, the J5 antiserum was shown to give broad protection against different Gram-negative organisms. Rabbits given the J5 antiserum also cleared labelled bacteria more rapidly than those given normal sera.

Chedid and coworkers found that administration of high titre antiserum prepared in a horse immunized with a rough S.typhimurium strain, TV 119, protected mice infected with a highly virulent strain of K.pneumoniae[179]. The protective effect of this antiserum was removed by absorption with rough organisms but not by smooth strains of S.typhimurium or S.typhi. Also, provided the Klebsiella were preincubated in normal mouse serum bactericidal activity could also be demonstrated in vitro. These authors had previously observed that viable klebsiella organisms recovered from the liver of endotoxin-stimulated mice and reinoculated to normal recipients were cleared very rapidly "as if they had been modified into a rough strain" (they had found that in contrast to the O antigen, rough endotoxin is cleared very rapidly from the blood of normal mice). In

view of these and other observations Chedid proposed that host serum enzymes are capable of removing smooth determinants from the surface of Gram-negative bacilli thus exposing R antigenic sites common to many bacterial strains and species and so providing a mechanism which enabled antibodies to rough determinants to protect against smooth bacteria. The system was seen as a primordial defence mechanism which phylogenetically predated the evolution of type specific immunity.

There was now considerable evidence to show that antibodies to rough determinants might protect against infections with heterologous smooth bacilli and McCabe suggested "that the cross protection induced by immunization with rough bacilli might prove clinically useful"[180]. He studied the effect of active and passive immunization of mice with various rough mutants of S.minnesota on experimental bacteraemia from heterologous Gram-negative bacilli.

The rough mutants Ra 60, Rb 345, Rc 5, $Rd_1$7, $Rd_2$3 and Re 595 of S.minnesota and the parent smooth strain S 218 were used in McCabe's experiments. Figure 9 below shows the chemical structure of the lipopolysaccharides derived from these organisms.

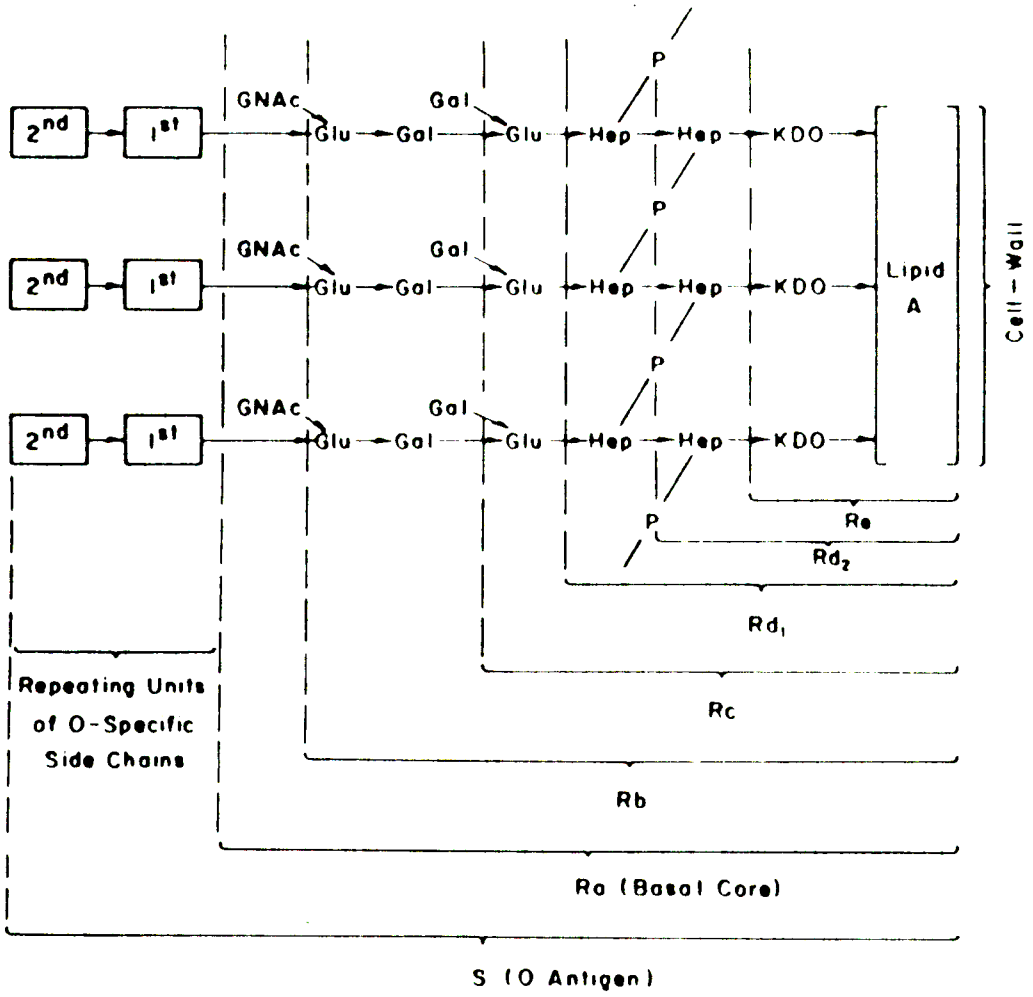

Figure 9. Chemical structure of lipopolysaccharides derived from cell walls of of S.minnesota S 218 and its rough mutants, Ra 60, Rb 345, Rc 5, Rd_1 7, Rd_2 3 and Re 595[180] . (GNAc, N-acetylglucosamine; Glu, glucose; Gal, galactose; Hep-P, heptose phosphate; KDO, 2 keto-3-deoxyoctonate).

Active and passive immunization of mice with the Ra, Rb, Rc and Rd_1 mutants of S.minnesota failed to protect against challenge with K.pneumoniae. However, active and passive immunization with the Rd_2 and Re mutants afforded significant protection against challenge with K.pneumoniae. Passive immunization with Re and active immunization with Ra, Rc, Rd_2 and Re provided significant protection against challenge with E.coli 107. Immunization with Re afforded greater protection than any of the other mutants. The protection afforded by immunization with the Re mutant was compared to type specific

immunization. Active immunization with the Re mutant was found to be considerably less effective than type specific immunization, nevertheless McCabe suggested that there are situations "where the induction of moderate degrees of immunity against a large number of microorganisms might be of clinical value"[180].

Neutropaenic rabbits were protected against bacteraemia and death from two strains of E.coli and one Enterobacter aerogines by immunization with the Re mutant of S.minnesota[181]. During the course of these studies it was found that immunization with lipid A was not protective. The lipopolysaccharide of the Re mutant differs from lipid A only by the presence of a ketodeoxyoctonate (KDO) trisaccharide. It was therefore concluded from this and from in vitro studies that KDO, not lipid A, was the important cross-reactive antigen[181,182].

In 1973, Ziegler, Douglas and Braude reported for the first time that human antiserum could also confer "exceedingly potent" passive immunity to the effect of endotoxin[12]. Twenty one men each gave a pint of preimune blood and were later immunized with a single injection of S.typhimurium or E.coli O:111 heat killed cells which had been suspended in physiologic saline. Immune serum was collected two weeks later. 1ml antiserum given intravenously protected mice against a lethal intravenous dose of homologous endotoxin. The incidence of positive local Shwartzman reactions to homologous endotoxin was reduced from 100 to 38% by E.coli antiserum and from 92 to 35% by S.typhimurium antiserum. There was however no protection against heterologous endotoxin in either animal model. The failure of heterologous protection with smooth antisera was explained by the concept that "O" determinants conceal the common core of endotoxin. In view of the fact that, antiserum from animals immunized with "O"-deficient mutants had been shown to afford heterologous protection against some of the adverse effects of endotoxin, it was decided to

immunize human subjects with the "O"-deficient mutant vaccines.

Human antiserum obtained after J5 immunization was tested in a rabbit model[183]. Pseudomonas bacteraemia was produced in experimental animals made agranulocytic with nitrogen mustard. A broth containing 10^7 Pseudomonas aeruginosa was instilled into the conjunctival sac. Within 48 hours of inoculation the animals developed conjunctivitis, facial oedema, infarction of the eyelid, and overwhelming bacteraemia, leading to death in more than 90% of the animals. There was however a 42% survival rate among rabbits given human J5 antiserum (intravenously, 24 hours after eye inoculation with Pseudomonas) while only 10% of the animals which received control serum survived.

Davis and coworkers have also shown that J5 antiserum can neutralize meningococcal endotoxin, which has been found to be biochemically similar to enteric lipopolysaccharide[184]. J5 antiserum also prevented mice from lethality in experimental Haemophilus influenzae Type b infection[185].

McCabe and his coworkers measured antibody titres (mainly IgM) to O-specific antigens and to the Re determinant of rough bacilli in patients with Gram-negative bacteraemia[5,186]. They found that high titres of O antibody did not prevent development of bacteraemia and were associated with only a slight decrease in the frequency of shock and death. In contrast, both shock and death were 1/3rd as frequent among patients with high titres of Re antibody. These observations and previous animal studies suggested that enhancement of resistance to enterobacteria might be accomplished by immunization with shared cross-reactive antigens and therefore a double-blind controlled trial using J5 antiserum was undertaken by Braude and coworkers in patients severely ill with Gram-negative bacteraemia. Control serum was collected from healthy, adult male volunteers who two months later received subcutaneous injection of 5×10^9 heat-killed J5 E.coli in two sites, followed by two identical injections 48 hours later[13,14].

The immunization is reported to have "resulted in local swelling and tenderness in most men and in transient fever and malaise in a minority". 14 days after immunization, immune serum was collected.

37 patients received J5 antiserum while 46 had been given control serum. Both groups received 3 ml of serum/kg body weight. The groups were similar in age, severity of underlying disease and incidence of neutropaenia and they had been treated equally often with granulocytes and high dose corticosteroids. The J5 antiserum group had more pseudomonas bacteraemia and more profound shock before administration of serum. Five of 37(14%) patients who received J5 antiserum died as a direct consequence of bacteraemia compared to 12 of 46 (26%) patients given control sera. Mortality in the J5 group was about half that among controls. 18 patients experienced "profound shock", i.e. their hypotension required treatment with pressors for at least 6 hours. Of these cases, only 2 of 7 controls, or 29%, recovered from shock. In contrast, 9 of 11 (82%) patients given J5 antiserum recovered from shock. The number of patients is small; however the authors consider the difference to be significant ($p = 0,024$ by Chi-square).

On the basis of these favourable results the trial was expanded in order to establish the full significance of the data. A randomized controlled trial involving 304 patients was undertaken over a seven year period[15]. 212 patients were eventually included in the analysis and of these 103 had received J5 antiserum and 109 had received nonimmune serum. In the entire study the number of deaths in bacteraemic patients was 42 of 109 (39%) in controls and 23 of 103 (22%) in recipients of J5 antiserum. J5 antiserum was particularly effective in patients with hypotension, especially those in profound shock, as shown in table 6.

Table 6

Effect of J5 antiserum on mortality from Gram-negative bacteraemia[15].

	Treatment Group		P Value
	Nonimmune Serum	J5 Antiserum	
	deaths/total (percent)		
Patients with positive blood cultures:	38/100 (38)	29/91 (24)	0.041
With hypotention	33/66 (52)	20/62 (32)	0.028
In profound shock*	26/34 (76)	17/37 (46)	0.009
Patients with negative blood cultures†	4/9 (44)	1/12 (8)	0.080+
All patients	42/109 (39)	23/103 (99)	0.011
With neutropenia	13/41 (32)	5/29 (17)	0.179
Without neutropenia	26/68 (43)	17/74 (23)	0.012
With hypotension	38/74 (51)	21/71 (30)	0.008
In profound shock*	30/30 (77)	18/41 (44)	0.003

*Requiring pressors for more than six hours.

†Blood was drawn after antibiotics had been given.

+With Yates' correction for small cell frequencies.

From these results Ziegler and coworkers concluded that J5 antiserum substantially lowers mortality from Gram-negative bacteraemia. The authors suggest that, although both the IgM and IgG fractions of J5 antiserum protect against lipopolysaccharide in the Shwartzman reactions, it is the IgM which is more potent against infections[15].

Most of the studies leading to the use of the J5 antiserum in the clinical trials described above have emanated from A.I. Braude's laboratory. There have however been numerous other studies on the protective role of anti-LPS antibodies in Gram-negative bacteraemia and these will now be reviewed.

Of particular interest is the development of a polyvalent pseudomonas vaccine of value in controlling invasive pseudomonas infection. Infection with Pseudomonas aeruginosa is common in certain types of patients, notably those with extensive tissue damage, particularly following burn injury and those with cystic fibrosis or neoplasia[187]. Early pseudomonas vaccines were monovalent but since infection can be caused by a strain belonging to any of the 16 internationally recognized serological groups of P.aeruginosa multivalent vaccines were produced.

Jones and coworkers report the preparation of a polyvalent vaccine comprised of 16 component vaccines, each a lipid-protein-carbohydrate complex extracted from the surface of one of the 16 different serotypes of P.aeruginosa [188]. Healthy volunteers were given three weekly subcutaneous injections of the polyvalent vaccine (PEV-01)[189]. Jones and coworkers report that "apart from slight or moderate local reactions and a transient rise of temperature in some volunteers, there were no clinical, biochemical or haematological abnormalities in the vaccinated volunteers". Raised titres of antibody in theserum of volunteers given 0,5 - 1.0 RHD (manufacturer's recommended human dose) vaccine were shown against all of the 16 serotypes of Pseudomonas aeruginosa. Serum from vaccinated individuals was shown to protect mice against challenge by intraperitoneal injection of Pseudomonas aeruginosa P14, which killed 100% of unprotected controls. The vaccine was subsequently tested in controlled trials at two burns units, in Birmingham (United Kingdom) and New Delhi (India), in children and adults with burns more than 15% full skin thickness[190]. In New Delhi where the risk of death from pseudomonas septicaemia is high, 13 of 32 (40,6%) of the unvaccinated adults died while only two of 20 (6,6%) of the vaccinated adults died. In Birmingham, where pseudomonas septicaemia occurs less frequently than in New Delhi, the number of deaths were similar in the vaccinated and unvaccinated groups. None of the vaccinated patients in either trial showed blood cultures containing Pseudomonas aeruginosa and

vaccinees showed raised titres of protective antibodies (both IgG and IgM) and increased phagocytic activity against Pseudomonas.

A hyperimmune gamma globulin called pseudomonas immunoglobulin (PI) was prepared from the plasma of healthy volunteers vaccinated with the polyvalent vaccine,PEV-01[16]. The efficacy of PI against pseudomonas infection was compared with PEV-01 and combined PI/PEV-01 treatments in a controlled clinical trial in severely burnt patients in New Delhi. In children the mortality was lowest in those passively immunized. All the 18 children immunized with PI survived compared with a mortality of 21% in the unimmunized children. In adults the mortality rate of those receiving immunoglobulin or vaccine was 10% or 8,3%, respectively, compared with 36% in controls. Combined vaccine and imunoglobulin treatment gave rather less protection than vaccine alone. It is interesting to note that only small volumes of PI were necessary to reduce the risk of pseudomonas septicaemia; the dose-schedule for children was 0,2 ml PI by deep intramuscular injection for 3 consecutive days, starting on the day of admission to the hospital, while "adults" (patients > 13 years old) were given 0,5 ml instead of 0,2 ml.

Pollack and Young who measured serum antibodies to lipopolysaccharide in 52 patients with Pseudomonas aeruginosa septicaemia also found that high anti-lipopolysaccharide titres were associated with survival[191]. The principal antibodies against Pseudomonas are thought to be opsonins of the IgG and IgM immunoglobulin classes, but it is unclear which, if either, is more closely associated with protection. The antibodies are potentiated by the complement system, activated either through the "classical" or "alternate" pathways[192].

An antiserum against pseudomonas endotoxin significantly protected mice challenged with live pseudomonas cells[193]. The LD_{50} for animals treated with antiserum was increased by $10^{2,7}$ times as

compared with the control group.

The pyrogenic activity of LPS from Yersinia enterocolitica was neutralized by homologous antiserum[194]. The level of homologous neutralization was markedly higher than that of heterologous neutralization.

Small amounts of E.coli 055:B5 endotoxin injected intramuscularly into cows induced pyrogenic tolerance and an increase in the rate at which the serum killed a strain of E.coli[195].

Escherichia coli is by far the main cause of human pyelonephritis. Vaccination with formalin-treated cells of E.coli 06 protected rats against unilateral retrograde pyelonephritis due to homologous E.coli serotype[196]. The protection was manifested by less parenchymal destruction and shrinkage of the pyelonephritic left kidney and also by less secondary pyelonephritis and compensatory hypertrophy in the right kidney of vaccinated rats. Only one of the 151 rats in the vaccinated group died while of the 162 rats in the control group, 22 died. High levels of antibody to E.coli LPS were found in the vaccinated animals and are thought to have contributed to protection.

Mattsby-Baltzer and coworkers found that the oral immunization of rats with a live strain of E.coli O6K13HI protected them against experimental ascending pyelonephritis caused by the same strain[197]. Sera collected 1 week after infection from the immunized animals were found to have increased levels of IgG anti-O6 and IgM anti-K3 in comparison with the nonimmunized controls. In urine, there was an IgG antibody response to the O6 antigen. During a 12 month follow-up of the infections, the serum anti-O6 antibody levels remained high throughout the entire observation period[198]. These studies thus raise the question of whether vaccination ought to be considered for patients predisposed to chronic bacterial pyelonephritis.

Injection of sublethal quantities of endotoxins extracted from smooth and rough bacterial strains have been shown to interrupt pregnancy in mice[199]. Interruption of pregnancy is shown to be related to the toxic lipid A moiety and not to be sero-specific polysaccharide. Pregnant mice were treated with specific antisera obtained by immunization of mice, horses or rabbits with heat-killed bacteria[199]. Immune serum was found to protect mice from the abortifacient effect of smooth or rough LPS, this activity being lost after absorption of the antiserum with homologous bacteria. Antibodies to lipid A protected pregnant mice challenged with lipid A or LPS extracted from various organisms.

Research into cholera has shown that there is "correlation between the complement-mediated serum vibriocidal activity of individuals and their resistance to the clinic disease"[200]. It would therefore suggest that the most effective vaccines would be those with a high content of the antigens against which the vibriocidal antibodies are directed. Since Vibrio cholerae is a Gram-negative organism the vibriocidal antibodies are thought to be specific for LPS determinants. Neoh and Rowley attempted to establish whether vibriocidal antibodies are identical to anti-LPS antibodies. They found that the antibodies which protect against cholera are mainly those directed against the oligosaccharide determinants of LPS[200]. However, in addition to this, there are antibodies directed against protein antigens present in the cell walls.

Sera from 50 patients with gonorrhoea, 30 with non-specific urethritis and 80 blood donors were treated with mercaptoethenol and examined for antibodies against endotoxin from Gonococci[201]. The percentage of sera active in a haemagglutination test was much higher in the gonorrhoea group than in the controls. The antibodies against the endotoxin are reported to be those of the IgG class. Rice and Kasper found that a relatively protein free LPS preparation from Neisseria gonorrhoea was the major antigenic constituent responsible for the

development of bacterial antibody (IgG) in rabbits and in a patient who suffered disseminated infection[202]. Strains of Neisseria gonorrhoeae are thought to vary in their susceptibility to serum bacteriocidal activity and no specific protective role of gonococcal bacteriocidal antibody has been identified. It is however interesting to note the response to a polysaccharide vaccine of group A Neisseria meningitidis used in a field trial in Finland involving large numbers of children during an epidemic in the 1970's[203]. Antibody responses were good in children older than 18 months of age and the vaccine resulted in a decreased incidence of the disease.

Studies have been undertaken to measure the levels of antiendotoxin antibodies in the serum of healthy individuals as well as those suffering from various disorders. A radioimmunoassay was used to measure anti-E.coli antibodies in the sera of 11 healthy subjects and 72 patients with immunological or gastrointestinal disorders[204]. IgG and IgM specific antibodies predominated quantitatively over IgA specific antibodies. Surprisingly, in this study alone the mean antibody concentrations were not significantly different from normal in patients with gastrointestinal conditions such as nontropical sprue, regional enteritis, intestinal bacterial overgrowth and chronic liver diseases in which elevations might have been expected. Others however reported increased frequencies of occurrence and titres of serum agglutinating antibodies to intestinal bacteria (including some E.coli strains) in cirrhosis and chronic hepatitis[205,206]. Differences in sensitivities of techniques of antibody detection as well as differences in serotypes of E.coli used may account for the conflicting results. Brown and Lee also reported diminished concentrations of IgG and IgA class antibodies in 5 patients with chronic ulcerative colitis[204]. In a later study, sera from 30 patients with inflammatory bowel disease (16 with Crohn's disease and 14 with ulcerative colitis) were assayed for the presence of antibodies against 159 Escherichia coli O-antigens and compared with sera from 16 matched control subjects[207]. The majority of patients

with inflammatory bowel disease had agglutinating antibodies (IgG and IgM) to a higher number of Escherichia coli O-antigens and in higher titres than the control group. The authors suggest that previously only a few serotypes of E.coli were tested while in their study all 159 internationally recognized E.coli serotypes were included. Since various serotypes of E.coli are present in different subjects unless all the O-antigens are tested, negative results may be reported. The study did not however reveal any correlation between the number of E.coli agglutinins and the site and severity of the disease or type of therapy.

There are a number of endotoxic activities for which the lipid A component of LPS is thought to be responsible e.g. Shwartzman reaction, pyrogenicity and complement activation[208]. The question is therefore raised as to whether anti-lipid A antibodies could be elicited by immunization with lipid A. A lipid A vaccine was prepared by Galanos and coworkers, and animals immunized with this vaccine produced anti-lipid A antibodies (IgG and IgM)[209,210].

The vaccine was produced by mild acid treatment of a salmonella Re mutant. This treatment removed KDO radicals, leaving cells with lipid A on their surface. These cells were then coated with additional free lipid A. Rietschel and Galanos examined the protective role of lipid A antiserum in rabbits, using lipid A-induced fever and skin necrosis as test systems[211]. It was shown that lipid A antiserum after passive transfer, conferred significant protection against skin necrosis and against fever providing that test animals had been pretreated with endotoxin. In this respect, lipid A antiserum differs from antisera specific to the polysaccharide portion of lipopolysaccharides. Why pretreatment is necessary in lipid A antiserum-mediated protection is not understood. Lipid A-induced local Shwartzman reaction could also be prevented by lipid A antiserum.

Antibodies to lipid A have been shown to be common in the sera of healthy children and adults, suggesting that exposure to lipid A or LPS from intestinal bacteria results in an antibody response

detectable in serum[212]. Such responses may be boostered by infections. Girls with acute cystitis, acute pyleonephritis and asymptomatic bacteriuria showed significantly elevated levels if IgG antibodies to lipid A as compared with children with no history of urinary tract infection.

This review has highlighted the potential role of antibody therapy in the treatment of Gram-negative bacteraemia. In the clinical studies discussed, antibodies were raised by vaccination of healthy volunteers with heat-killed Gram-negative bacteria or vaccines containing LPS. Gaffin and coworkers have however found that approximately 7% of plasma units in a blood bank have significant concentrations of antiendotoxin antibodies[18]. These antibodies have been shown to protect cats from lethal endotoxic shock secondary to haemorrhage. Thus, by routinely screening plasma units in a blood bank and pooling those units high in antiendotoxin titres, stocks of antibody can be accumulated on a large scale. The last part of this literature review (2.7) examines the measurement of antiendotoxin antibodies by enzyme-linked immunosorbent assay (ELISA), a technique found to be suitable for assaying large numbers of serum samples. Various studies, by Gaffin and coworkers, on the use of their antiendotoxin antibodies are described in Chapter 5 (5.2 and 5.3).

2.7 MEASUREMENT OF ANTIENDOTOXIN ANTIBODIES BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The potential of ELISA for detecting antibodies to bacteria has not been fully exploited until recently. Nevertheless as far back as 1972 Carlsson and coworkers detected salmonella O-antibodies in human sera by ELISA[213,214]. When compared with the conventional tube agglutination method (Widal reaction), passive haemagglutination and quantitative precipitation, ELISA was shown to be more sensitive. In addition, IgG and IgM antibodies were both detected approximately as efficiently with ELISA. Both the passive haemagglutination method and the Widal reaction have an important disadvantage in that IgM antibodies are preferentually detected. This has led to confusion in subsequent studies evaluating the effectiveness and importance of

specific IgG. Moreover, the methods used in these studies were not designed for large-scale routine serology.

ELISA was used successfully to determine the immunoglobulin class and titres of specific antibodies to cholera exotoxin and endotoxin[215,216]. Titration of the antiendotoxin antibodies was done with techniques involving secondary antibody manifestations, like vibriocidal effects and agglutination, and compared to ELISA which measures the primary binding of antibodies to solid phase antigen. Similar titre values were registered with the vibriocidal and the agglutination techniques, whereas the ELISA, allowing separate determination of antibodies of different classes, gave titres from 10 to 1000 times higher[216]. In a more recent report an assay capable of detecting antibodies directed against LPS and non-LPS Vibrio cholerae surface antigens is described[217]. Sodium azide-killed whole cells of V.cholerae were adsorbed to polystyrene tubes and used as immobilized antigens.

Local antibody formation by plasma cells in E.coli infected rabbit kidneys has been studied by the ELISA technique [218]. Antibodies against E.coli O antigen, which developed in response to immunization of rabbits with formalin-killed bacteria, were also measured by ELISA[219]. Ahlstedt and coworkers have in fact found the ELISA to be a most useful method in determining antibodies against various E.coli antigens. Using the ELISA, they were able to measure antibodies in the urine of patients with acute pyelonephritis[220].

After the successful use of ELISA for determining antibody to smooth LPS, the method was also applied to measure antibody to rough LPS. The technique was found to be more sensitive than quantitative precipitation in determining IgG antibodies directed to Salmonella minnesota Re glycolipid[221,222]. Ito and coworkers attempted to duplicate the work of Bruins and his asociates[221] to measure antibodies to the rough LPS of E.coli J5[223]. They were unsuccessful and instead found that the rough endotoxin was not adhering in sufficient amounts to the surfaces of the polystyrene tubes used in the assay. They were however successful when antigen adherence was

carried out in the presence of 0,02M magnesium chloride. It is not known how magnesium ions mediate the adherence of rough J5 endotoxin to polystyrene. Gonococcal LPS was also found to require magnesium ions in order to adsorb to polystyrene.

ELISA has recently been found to be a sensitive and highly reproducible method of measuring anti-lipid A antibody[22,225]. The technique was used to measure antibodies to lipid A in the serum of patients with urinary tract infections[212].

ELISA is reported to be an excellent method of screening populations for brucella antibodies[226]. Assays for total anti-brucella antibodies (IgG + IgM + IgA) in the sera of patients with brucellosis gave readings that were more than double those found in hundreds of control sera.

In the studies discussed so far, the ELISAs were carried out in polystyrene tubes. However, in situations where large numbers of samples are to be assayed, a microtitre ELISA is more appropriate. Young and coworkers describe a microtitre assay for measuring immunoglobulin G cholera antitoxin in humans serum[227,228]. The assay was found to be specific, sensitive and reproducable and only needed as little as 5 ul of serum. The method is said to be particularly well suited to seroepidemiological surveys. A microtitre ELISA was also used to measure group B streptococcal antibodies (IgG)[229].

In conclusion, it is hoped that ELISA by giving more sensitive measurements of antibodies will lead to a greater understanding of the role of antiendotoxin antibodies in immunological resistance to Gram-negative infections.

CHAPTER 3

MATERIALS AND METHODS

3.1 MEASUREMENT OF ANTIENDOTOXIN ANTIBODIES IN HUMAN SERUM BY ELISA

3.1.1 Introduction

The indirect ELISA was used for the measurement of antiendotoxin antibodies in human serum. The basic procedure is outlined below:

(a) Antigen is attached to the solid phase and then washed.

(b) Test serum, diluted as required, is added and incubated at a standard temperature and time. This facilitates the attachment of any specific antibodies to the antigen. This is followed by washing.

(c) Enzyme labelled anti-antibody is added and allowed to react with the treated plate and then rinsed.

(d) Substrate to the bound enzyme is added. Degradation of substrate results in a colour change. The reaction is stopped after an appropriate period of time and the amount of colour change is related to the amount of antibody in the test sample.

Human serum samples were provided by the Natal Blood Transfusion Service from blood units donated by volunteers in the Natal Province. Before use, the serum was centrifuged for 5 minutes at 16 000 rpm and then diluted 1:50 with 0,9% sodium chloride solution. Saline solutions and water used throughout the study were sterile and pyrogen free (Sabax Laboratories, Johannesburg). Only Analar reagents were used in the preparation of buffer solutions.

3.1.2 The binding of endotoxin to microtitre plates

Endotoxins (Difco Laboratories, Detroit) from the following 12 bacterial strains and species were used : E.coli 055:B5, E.coli 0127:B8, E.coli 0128:B12, E.coli 026:B6, E.coli 0111:B4, Sh. flexneri, S.minnesota 9700, S.abortus equi, S.marcescens, S.typhosa, S.typhimurium and S.enteritidis.

A mixture of endotoxins was used to coat microtitre plates according to the materials and methods section of the publication: Gaffin, S.L., Badsha, N., Brock-Utne, J.G., Vorster, B.J. and Conradie, J.D. An ELISA procedure for detecting human anti-endotoxin antibodies in serum[230]. A copy of this publication is appended to this thesis.

3.1.3 ELISA technique

(a) 50 µl of the diluted serum samples were applied to the wells of the coated microtitre plates. Serum samples were also introduced into blank wells. The plates were incubated for 1 hour at 45°C.

(b) The plates were then emptied, washed with TST buffer, pH 8,0 (0,05 M tris-0,1M NaCl with 0,02% Tween 20 and 0,02% sodium azide, NaN_3, as a preservative) and dried.

(c) Conjugate consisting of sheep anti-human antiendotoxin antibodies and horseradish peroxidase was prepared according to the method of Wilson and Nakane[231] by the Natal Blood Transfusion Service. It was diluted (usually between 1:50 and 1:100 depending on the batch of conjugate used) in saline and 50 µl were pipetted into each well of the microtitre plates and left to incubate for 1 hour at 45°C.

(d) The plates were then emptied and rinsed as in (b) above.

(e) 50 µl chromogenic substrate were pipetted into each well. Colour was allowed to develop for 30 minutes, in the dark, at room temperature. Chromogenic substrate was made up of 18,4 mM $Na_2B_4O_7.10H_2O$ (Borax, 0,70g), 30,5 mM succinic acid (0,36g), 3,7 mM O-phenylene diamine (40mg), 4,0 mM urea hydrogen peroxide (40 mg) and distilled water to 100 ml[232]. It was provided by the Natal Blood Transfusion Service.

(f) The enzymic reaction was stopped by adding 150 µl 1 N hydrochloric acid to each well.

(g) The intensity of colour developed in each well at 492 nm was measured using a photometer (Vitatron DCP, Holland).

3.2 IMMUNOPRECIPITIN TECHNIQUE

Serum endotoxin-specific antibodies were also measured by an immunoprecipitin procedure, adapted from Gaffin and coworkers[18].

(a) Cyoprecipitates were removed from serum samples by centrifuging the samples at 7 000 rpm for 30 minutes, incubating them overnight on ice and centrifuging them again, to clarify, at 7 000 rpm for 30 minutes, before use.

(b) A mixture of endotoxins obtained from 5 bacterial strains (E.coli 055:B5, S.minnesota 9700, S.typhimurium, S.typhosa and S.enteritidis; Difco) were dissolved in water at a final concentration of 1 mg/ml.

(c) 300 µl of the serum samples were added to 100 µl of the endotoxin mixture and 10 µl 1M phosphate buffer (NaH_2PO_4/Na_2HPO_4), pH 7,3. Controls were set up in which serum samples were added to 100 µl distilled water and 10 µl 1M phosphate buffer, pH 7,3. All

samples were tested in triplicate. The solutions were incubated for 2 hours at 37°C and then left overnight at 4°C.

(d) The solutions were then centrifuged in the cold for 20 minutes at 16 000 rpm and the supernatant fractions were discarded.

(e) The precipitates were washed twice with 1 ml ice-cold phosphate buffered saline, PBS (0,8% NaCl, 0,02 M NaH_2PO_4/Na_2HPO_4)pH 7,3.

(f) The protein was then determined using a Coomassie blue stain assay[233]. 0,5 ml Coomassie blue reagent* was added to each of the precipitates. The intensity of the colour which developed after leaving the samples at room temperature for 20 minutes, was read in a photometer at 600 nm. The amount of protein present was then determined from a standard curve obtained by adding bovine serum albumin, BSA, (0 to 200 µg) to Coomassie blue reagent.

*Preparation of Coomassie Blue reagent

50 mg of Coomassie blue (Brilliant blue G, Sigma, St.Louis) was added to 25 ml ethanol (95%) and mixed for 1 hour after which 50 ml phosphoric acid (85%) was added and the solution was stirred well and filtered before use.

3.3 EXTRACTION OF BACTERIAL ENDOTOXINS

3.3.1 Introduction

During the latter half of the study it was found that commercially prepared endotoxin from Shigella flexneri was no longer available. It therefore became necessary to extract the endotoxin in our laboratory, using the basic phenol-water procedure described by Westphal and coworkers[33]. Some modifications suggested by other workers were

incorporated into the method which is described below[34]. The basic principle of the method is that proteins and lipoproteins are dissolved in the phenol phase while the upper water phase contains the liberated lipopolysaccharides and polysaccharides together with nucleic acids.

3.3.2 Cultivation and harvesting

A stock culture, made up of a mixture of Shigella flexneri strains, was obtained from Professor H.J. Koornhoff of the South African Institute of Medical Research, Johannesburg. This stock was cultured, by B.J.Vorster of the Blood Transfusion Service, Pinetown, in a trypticase soy broth (Biolab, Pretoria) for 5 days at 35°C, with constant agitation. The bacteria were killed by autoclaving and harvested by centrifugation for 30 minutes at 3 000 rpm.

3.3.3 Extraction procedure

(a) 60 ml distilled water were added to 10 g (wet weight) of bacteria. The suspension was brought to 70°C with constant stirring.

(b) After a homogenous suspension had been obtained, 60 ml 90% phenol, which had been preheated to 70°C, were added.

(c) The mixture was stirred for 15 minutes at 70°C and the solution was distributed to glass centrifuge tubes, which were then chilled in an ice bath overnight to allow the phases to separate.

(d) The tubes were centrifuged at 5 000 rpm for 15 minutes at 4°C.

(e) The aqueous supernatant phases were saved and 60 ml distilled water were added to the combined phenol phases and the extraction was repeated as described in (c) and (d) above. The second

aqueous phase was combined with the first, and the phenol residue was discarded.

(f) The aqueous phase was dialyzed at 4°C for at least 24 hours against several charges of distilled water.

(g) The solution was then concentrated at 4°C, by an ultrafiltration technique, in the dialysis bag, to approximately 1/9th of its original volume using Aquacide II (sodium salt of carboxymethylcelluslose, Calbiochem-Behring Corporation, Lucerne).

(h) To this concentrated solution at 4°C was added dropwise with stirring 60 ml pre-cooled methanol, which contained 1% volume of 20% magnesium chloride in ethanol. After the suspension was left overnight at 4°C, the precipitate was collected by centrifugation at 10 000 rpm for 10 minutes and dissolved in 20 ml cold distilled water.

(i) To this solution at 4°C was added dropwise with stirring 40 ml cold methanol, which contained 0,1% volume of 20% magnesium chloride in ethanol. The precipitate was collected as described in (h) above and dissolved in 12 ml distilled water.

(j) The endotoxin solution was frozen and lyophilized.
The endotoxins extracted were found to be protein-free, as assayed by the Coomassie blue procedure.

3.3.4 Assessment of endotoxin activity

Assays of the activity of each batch of Sh.flexneri endotoxin were carried out:-
When serum high in antiendotoxin antibody (as determined by ELISA) was preincubated with the prepared Sh.flexneri endotoxin (50 µg/ml) at 37°C for 30 minutes and then assayed, the ELISA was significantly reduced.

In addition, test plates were coated with different concentrations (1, 10, 30, 100 and 300 µg/ml) of the prepared Sh.flexneri endotoxin and the ELISA was carried out with serum high in antiendotoxin antibodies. The intensity of the colour was found to depend on the concentration of endotoxin coating the wells.

3.4 RELATIVE ACTIVITY OF LG-1* ANTIBODIES

LG-1 was diluted 1:800 in 0,1M NaCl containing 0,05 M Tris adjusted to pH8,0 and also containing 2% each of sheep and bovine serum (obtained from the Natal Blood Transfusion Service). The assay was carried out on microtitre plates coated with only one of each of the following endotoxins separately: E.coli 055:B5, E.coli 0127:B8, E.coli 0128:B12, E.coli 0111:B4, E.coli 026:B6, S.minnesota, Sh.flexneri, S.abortus equi, S.marcescens, S.typhosa, S.typhimurium and S.enteritidis. Several wells on every plate were coated with LPS from Sh.flexneri to use as a reference standard. The plates were incubated with 1% bovine serum albumin, BSA, (100 µl/well) at 37°C for 1 hour, rinsed with 500 ml TST buffer and dried before being used in the ELISA.

*Plasma units with high concentrations (>40 µg/ml) of antiendotoxin IgG were pooled and fractionated, by the Natal Blood Transfusion Service, to obtain a gamma globulin - Lot LG-1.

3.5 DIFFERENTIAL ABSORPTION EXPERIMENTS

LG-1 was preincubated with a variety of endotoxin-containing solutions and then tested for antibody activities remaining. LG-1 was diluted 1:800 in 0,1M NaCl containing 0,05M Tris adjusted to pH 8,0 and also containing 2% each of sheep and bovine serum. This solution was used in the following incubations all carried out at 37°C for 10 minutes, after which the samples were stored on ice. In each case endotoxin was added to a final total concentration of 50 µg/ml.

(a) The diluted LG-1 was incubated separately with each one of the following endotoxins: E.coli 055:B5, E.coli 0127:B8, E.coli 0128:B12, E.coli 0111:B4, E.coli 026:B6, S.minnesota, Sh.flexneri, S.abortus equi, S.marcescens, S.typhosa, S.typhimurium and S.enteritidis.

(b) Diluted LG-1 was incubated with a mixture of all twelve endotoxins, each at a concentration of 4,16 µg/ml.

(c) Diluted LG-1 was incubated with mixtures of eleven of the above twelve endotoxins by omitting each one in turn.

(d) As controls the diluted LG-1 was incubated with 0, 1M sodium acetate buffer, pH 5,0.

The above absorbed LG-1 samples were centrifuged at 4°C for three minutes at 16 000 rpm. They were then assayed for antiendotoxin antibodies by means of the ELISA procedure previously described. The assay was carried out on two types of microtitre plates: those coated with only one of each of the above endotoxins separately, and those coated with a mixture of all 12 endotoxins. The plates were incubated with 1% bovine serum albumin, BSA,(100 µl/well) at 37°C for 1 hour, rinsed with 500 ml TST buffer and dried before being used in the ELISA.

Preliminary experiments had been conducted in order to establish the optimal time required for preincubation of LG-I with endotoxin. LG-I was preincubated with endotoxin for periods ranging from 15 seconds to 5 hours; it was found that the reaction occurred within seconds and had reached conclusion by 10 minutes.

Similarly, LG-1 was preincubated with different endotoxin concentrations (1, 10, 30, 50 and 75 µg/ml) before it was established that the optimal endotoxin concentration for preincubation was 50 µg/ml.

CHAPTER 4

RESULTS

4.1 SPECIFICITY OF ELISA FOR ANTIENDOTOXIN ANTIBODIES

Three high-titre serum samples (as determined by ELISA) were preincubated with a mixture of endotoxins (E.coli 055:B5, S.minnesota, S.typhimurium, S.typhosa and S.enteritidis) at a final concentration of 10 µg/ml and 100 µg/ml, for 1 hour at 37°C. When subsequently assayed, the ELISA was either greatly reduced or negative for serum preincubated with endotoxin at both concentrations. The results are shown in table 7.

It is assumed that the antiendotoxin antibodies present in these samples were bound to the added endotoxins and were therefore unavailable to bind to the endotoxin coating the microtitre plates, hence, the reduced colour development.

Table 7

Effect, on ELISA colour development, of preincubation of serum with endotoxin

Serum sample No.	Δ OPTICAL DENSITY 492nm.		
	Untreated Serum	Serum preincubated with 10µg/ml endotoxin	Serum preincubated with 100µg/ml endotoxin
2	0,627	0,000	0,007
20	0,480	0,081	0,000
48	0,836	0,168	0,012

4.2 ENDOTOXIN COATING OF MICROTITRE PLATES

A solution of a mixture of endotoxins (E.coli 055:B5, S.minnesota, S.typhimurium, S.typhosa and S.enteritidis), at the following concentrations, 0,001; 0,01; 0,1; 1,0; 10,0; and 100 µg/ml, was adsorbed on to the surfaces of the wells of the microtitre plates. Three high titre serum samples (as previously determined by ELISA) were assayed at each of the above endotoxin concentrations, using the standard ELISA technique. The intensity of the final colour was found to depend on the concentration of endotoxin coating the wells.

For all three samples, at endotoxin concentrations less than 10 µg/ml the colour intensity was low, but at 10 µg/ml and higher, the optical density increased substantially, as shown in Figure 10. For reasons of economy, all subsequent experiments in this study were carried out at an endotoxin concentration of 10 µg/ml.

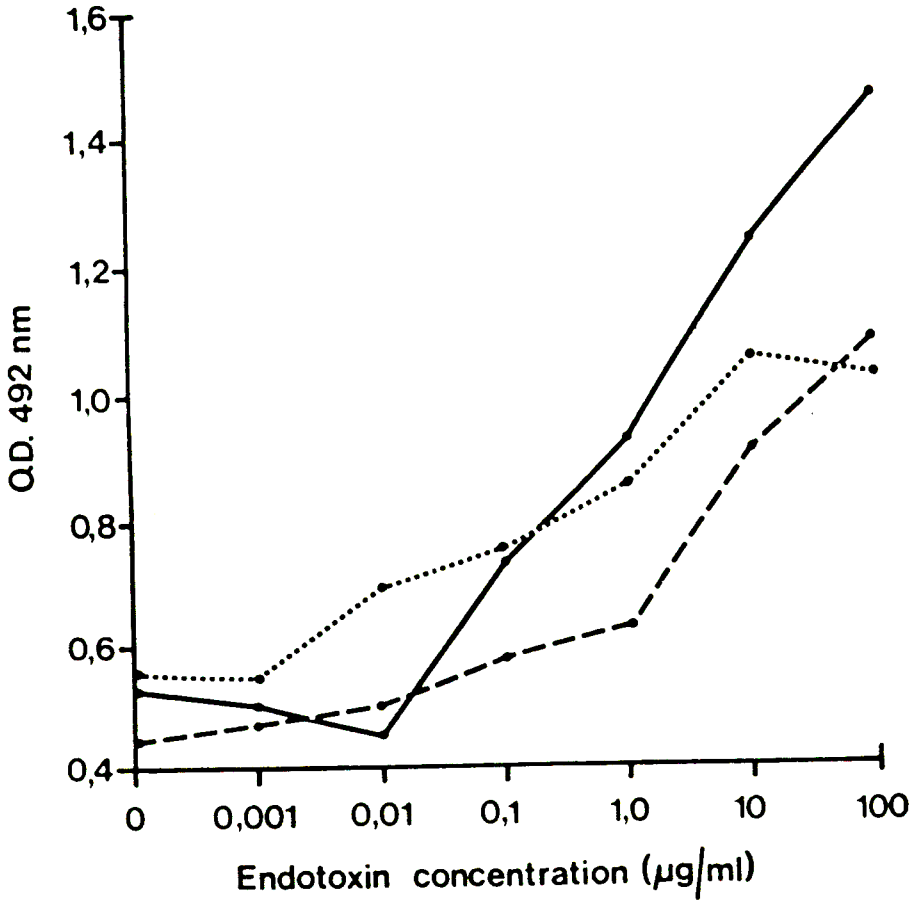

Figure 10. Effect, on ELISA colour development, of varying endotoxin concentration.

4.3 CALIBRATION OF THE ELISA

Serum sample number 104 was found to have an antiendotoxin antibody concentration of 44,4 µg/ml according to the immunoprecipitin technique described on page 68. Sample 104 was then diluted with 0,1M sodium chloride and tested by ELISA at various concentrations, as shown in Figure 11.

The optical density curve was divided into four regions for easy visual evaluation as 0, +, ++, and +++, which correspond to antiendotoxin antibody concentration ranges of 0 - 2,5 µg/ml, 2,5 -5 µg/ml, 5 - 40 µg/ml, and greater than 40 µg/ml, respectively.

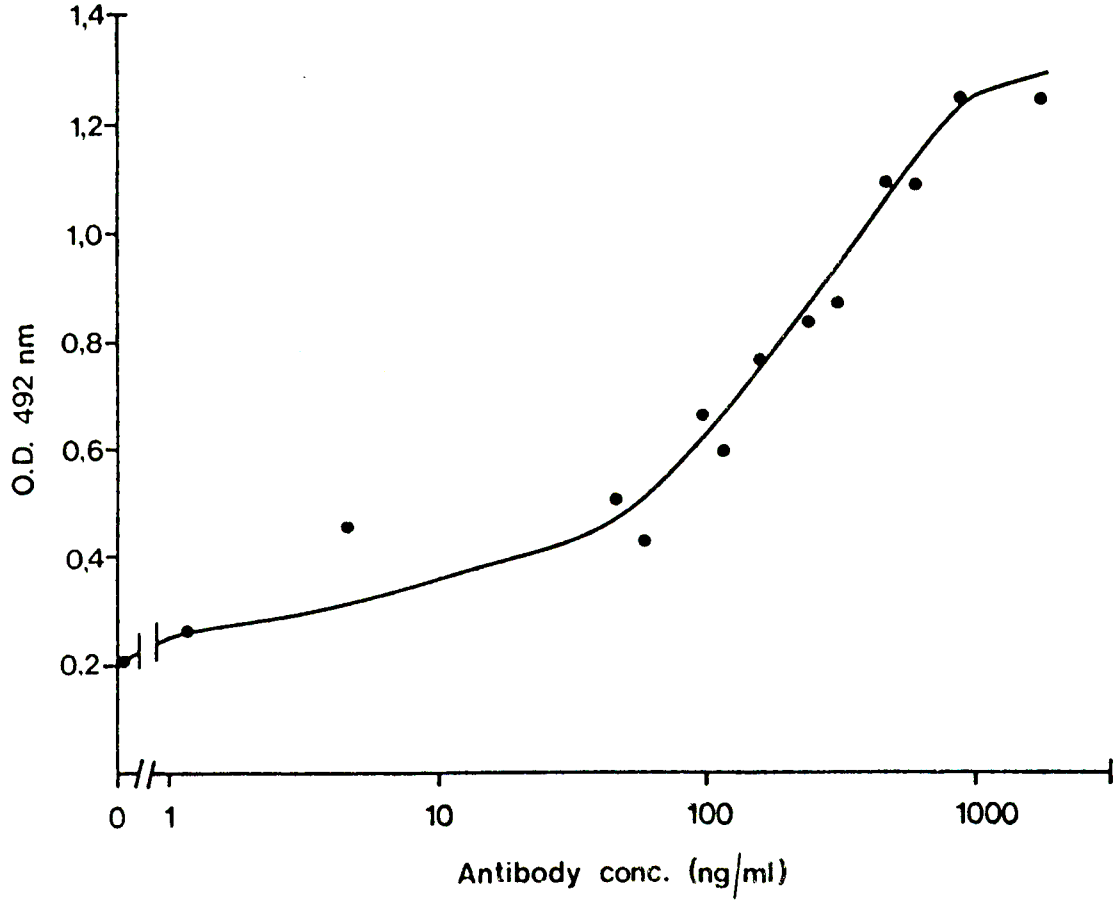

Figure 11. Calibration of the ELISA.

4.4 DISTRIBUTION OF ANTIBODY CONCENTRATION

Sera from 48 blood units (supplied by the Natal Blood Transfusion Service) were screened for antiendotoxin antibodies using the ELISA described on page 67. Subsequently, large numbers of serum samples were similarly assayed by Mrs Maureen Rome in our laboratory. All blood units donated to the Natal Blood Transfusion Service are now routinely screened for antiendotoxin antibodies at their Pinetown ELISA laboratory.

Figure 12 shows the distribution of antiendotoxin antibody titres among 1051 blood units[230]. 42% of the units tested had antiendotoxin antibody concentrations of zero to 2,5 µg/ml, 31,7% had 2,5 - 5 µg/ml, 16,8% had 4 - 40 µg/ml and 8,3% had a antiendotoxin antibody concentration greater than 40 µg/ml. Plasma units with concentrations of antiendotoxin IgG above 40 µg/ml are pooled and fractionated to obtain gamma globulin. It had previously been found that antibody concentrations of 40 µg/ml and above are therapeutically useful in cats[18]. One technician working with automatic pipetting and optical reading equipment is able to screen 200 serum samples per day or 50 000 per year. With an effective recovery of 7% this provides 3500 units or approximately 700 litres of high-titre plasma per year (B.J. Vorster, pers. comm*).

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*Mr B.J. Vorster, Natal Blood Transfusion Service, Pinetown

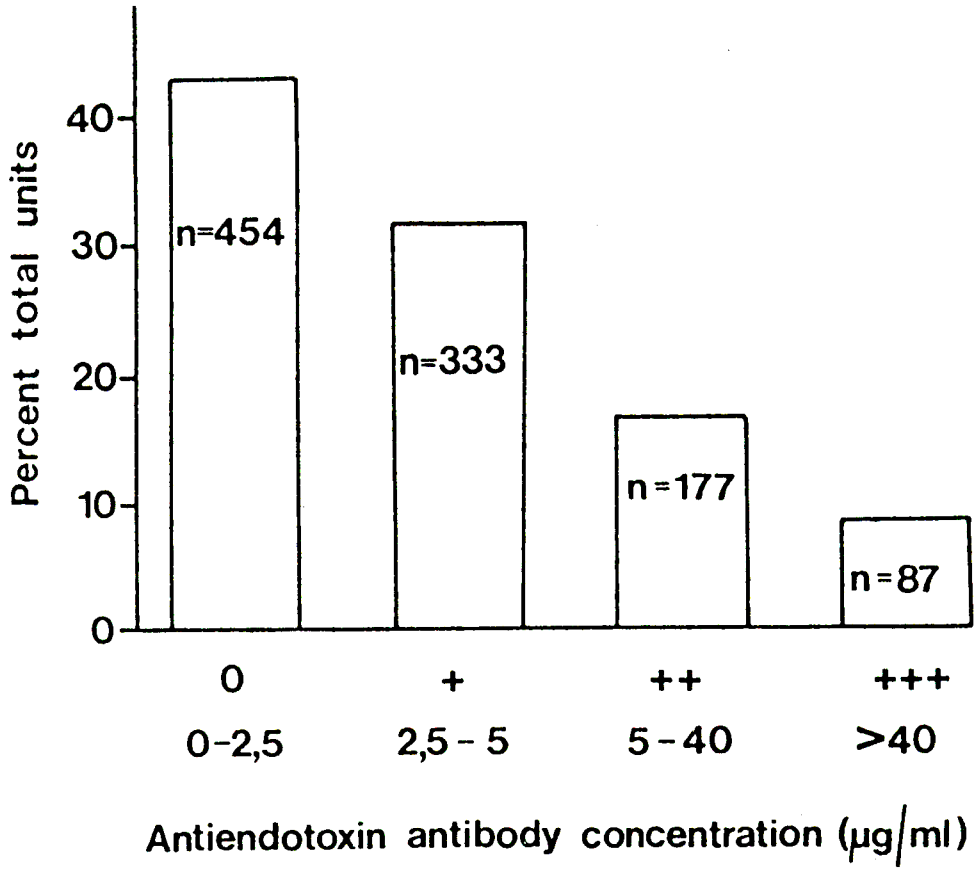

Figure 12. Distribution of antiendotoxin antibody concentrations in 1051 plasma samples [234].

In Natal, blood units containing high concentrations of specific antibodies (>40 µg/ml) were found with a frequency of 3,6% among all white donors (n = 1658) and 10,35% among all African donors (n = 2482, X^2 = 55,4, p < 0,01)[234]. The White male and female donors had no significant differences between them but the African women had almost twice the frequency of high titre serum as the African men (14,4% versus 7,6%, respectively, X^2 = 29,1, p < 0,01)

It was then decided to investigate the levels of serum antiendotoxin antibodies in Natal Indians to see if there were significant differences between the sexes. 148 serum samples obtained from the Chemical Pathology Laboratory of the R.K. Khan Hospital in Durban were screened for antiendotoxin antibodies. These samples were all from Indian hospital patients.

Indian women patients were not found to have a higher frequency of high titre serum than the Indian male patients. 10/97 female patients and 3/51 male patients had serum antiendotoxin antibody concentrations above 40 µg/ml (10,3% versus 5,9% respectively, x^2 = 0,82, p = 0,25). The distribution of antiendotoxin antibody concentrations in the serum of these patients is shown in Figure 13.

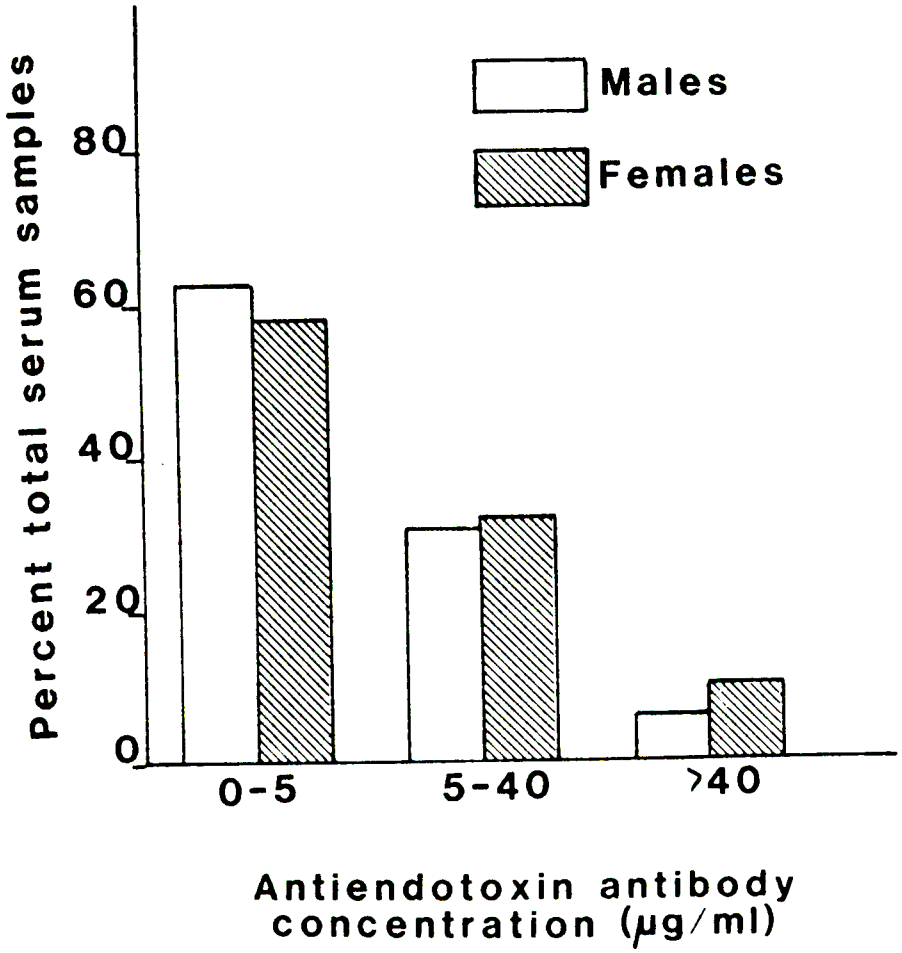

Figure 13. Antiendotoxin antibody levels in male and female patients at R.K. Khan Hospital, Durban.

4.5 SPECIFICITY OF HIGH TITRE SERA FOR VARIOUS ENDOTOXINS

Microtitre plates were coated with individual endotoxins (10 µg/ml) obtained from 12 different bacterial species and strains. Serum samples previously found to be high in antiendotoxin antibody titres (>40 µg/ml) were tested by ELISA on these plates. The height of each bar in Figure 14 represents the antibody concentration measured in 12 serum samples. Each sample was found to have its own characteristic specificities. Most samples were found to react strongly with S.typhimurium, S.enteritidis and Sh. flexneri but poorly with S.marcescens, E.coli O111:B4, E.coli O27:B8 and S.minnesota.

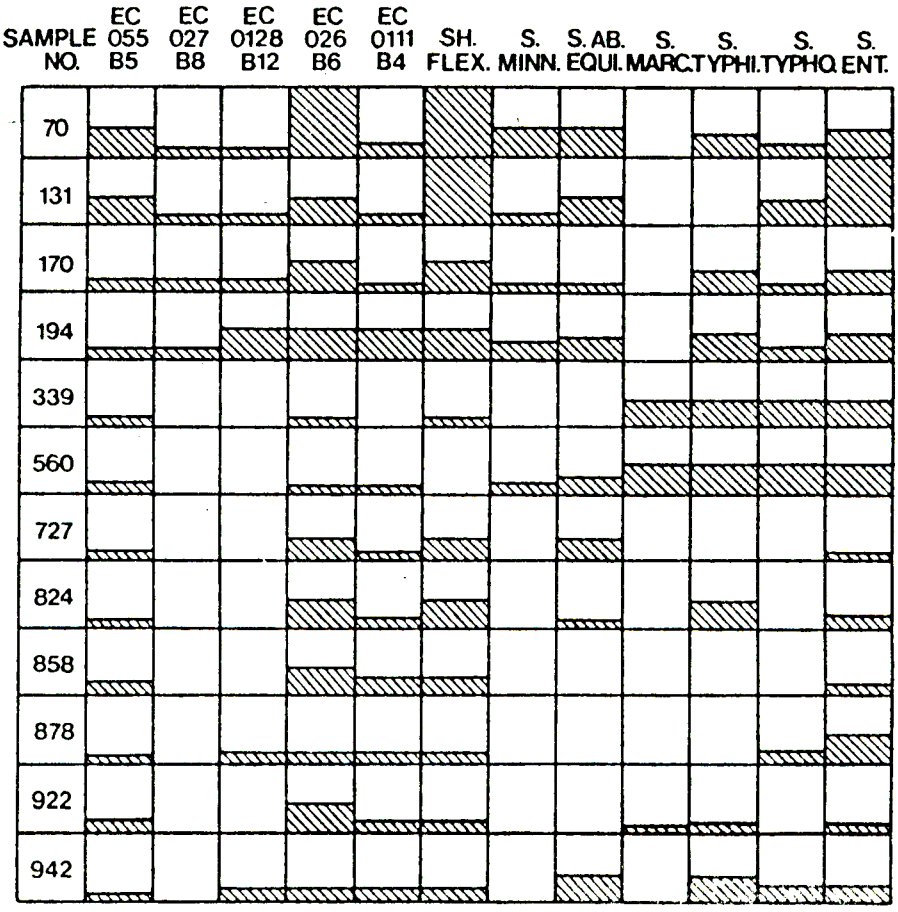

Figure 14. Specificities of antiendotoxin antibodies in 12 high-titre serum samples.

4.6 RELATIVE ACTIVITY OF LG-1 ANTIBODIES

The ELISA colour development, and, hence, the binding of LG-1 antibodies to 14 different endotoxins is shown in Figure 15. Binding was found to be highest with endotoxin from Shigella flexneri, S.abortus equi and S.typhimurium, intermediate with S.enteritidis E.coli 026:B6, K.pneumoniae and P.aeruginosa. Binding with the other endotoxins was relatively low but reproduceable.

The activity of the LG-1 antibodies to endotoxin from K.pneumoniae and P.aeruginosa was determined by B.J. Vorster of the Natal Blood Transfusion Service[235].

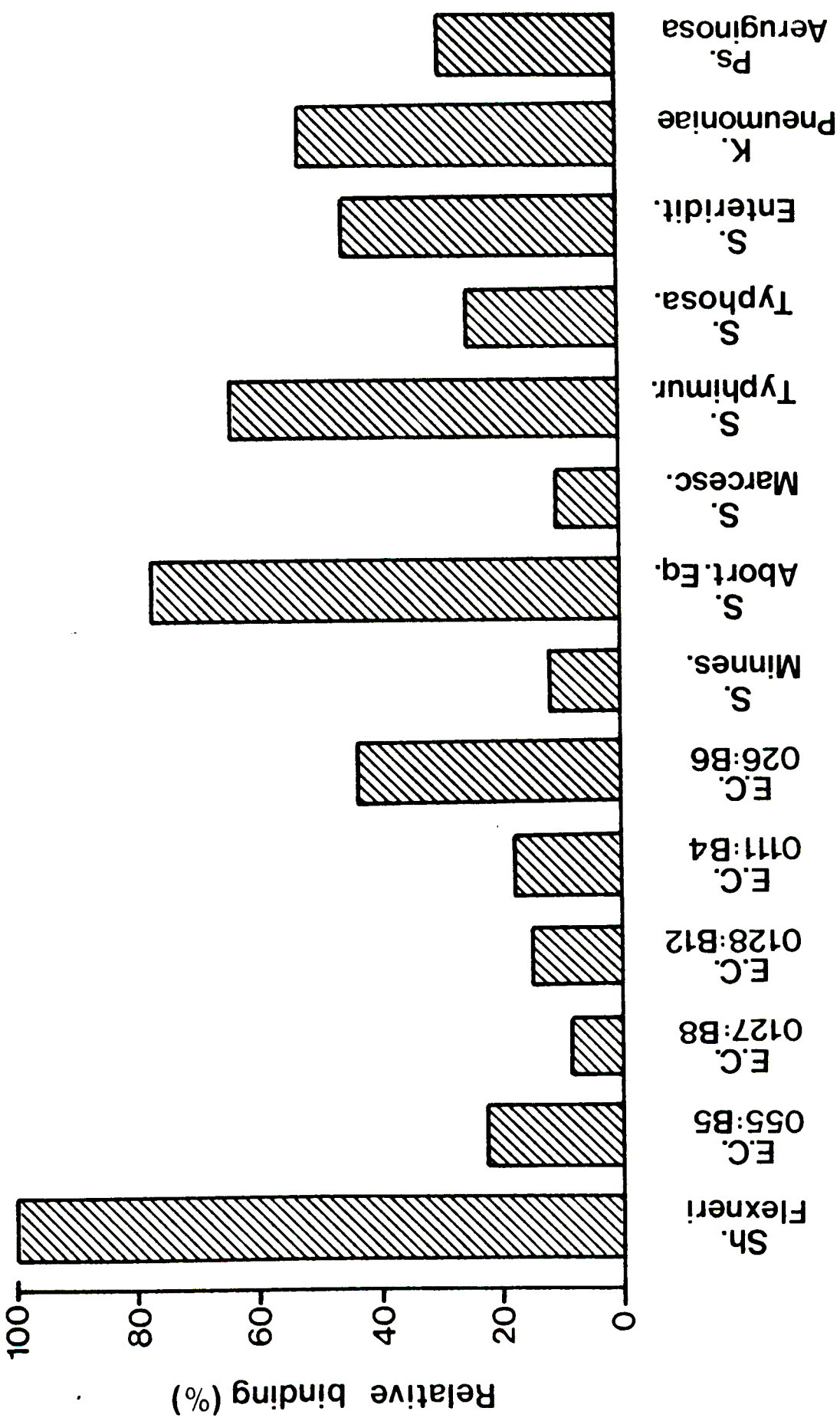

Figure 15. The relative binding capacities of LG-1 antibodies to endotoxins from 14 bacterial species and strains.

4.7 COMPARISON OF LG-1 ANTIBODY ACTIVITY TO THAT OF NORMAL HUMAN IMMUNOGLOBULIN

The activity of the LG-1 antibodies was compared with that of normal human immunoglobulin preparations obtained from the National Blood Fractionation Centre, Pinetown. Two gamma globulin fractions were tested, standard globulin 1 (Batch G-25, manufactured October, 1981) and standard globulin 2 (Batch G-27, manufactured, November, 1981). They were diluted and assayed in the same manner as the LG-1 preparation. The results, shown in Figure 16 indicate that the antibodies in the standard gamma globulin preparations bind to most of the endotoxins tested but to a considerably less extent than the LG-1 antibodies. The binding capacity of the antibodies in the standard globulin preparations towards most of the endotoxins tested was less than 15% of that of the LG-1 antibodies.

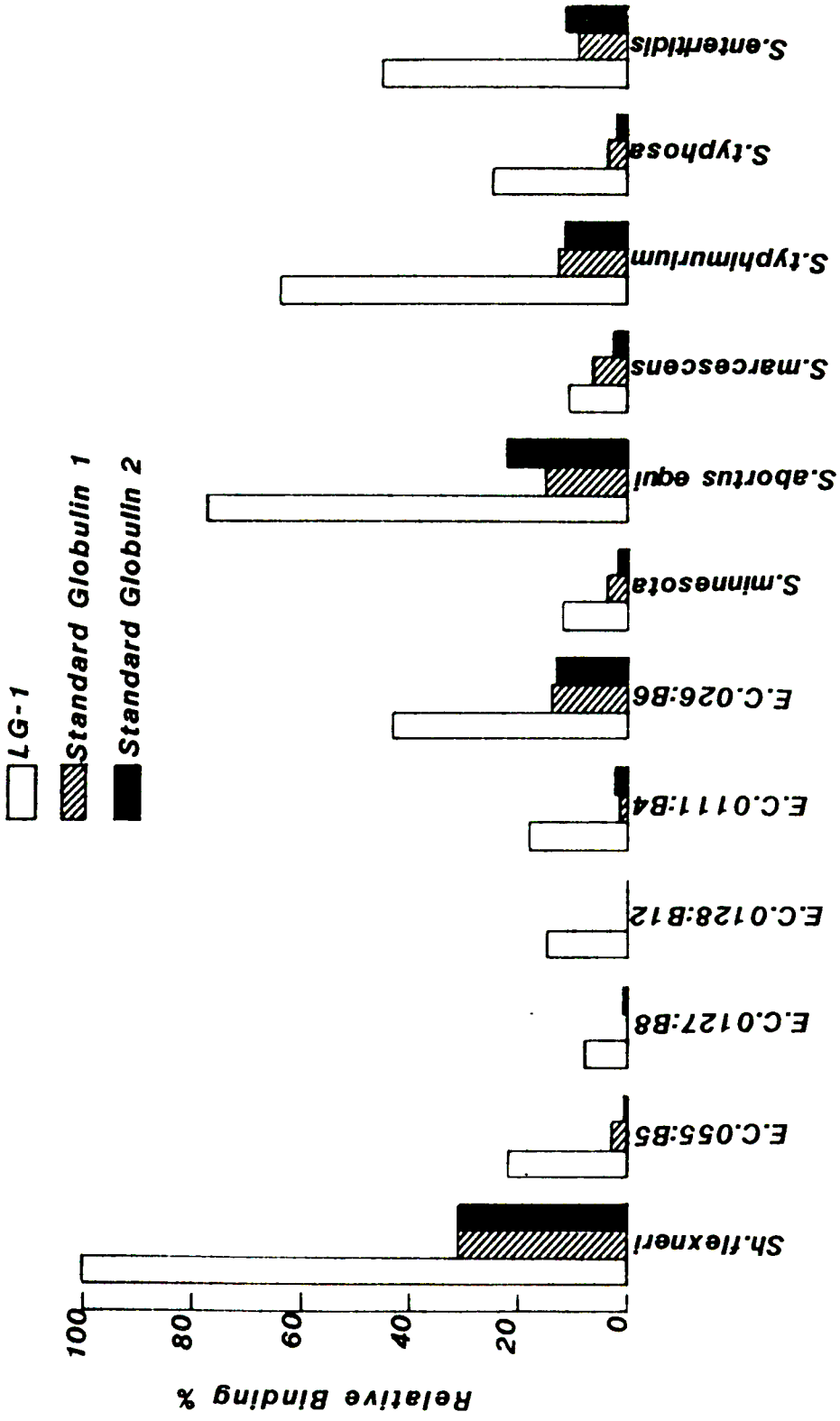

Figure 16. Comparison of LG-1 with standard globulin preparations.

4.8 COMPARISON OF LG-1 ANTIBODY ACTIVITY TO THAT OF AN ANTI-PSEUDOMONAS IMMUNOGLOBULIN, GX-9

The activity of the LG-1 antibodies was similarly compared with that of an anti-pseudomonas immunoglobulin fraction, GX-9, prepared by Wellcome Laboratories, England.

The results, shown in Figure 17 clearly show that the binding capacity of the LG-1 antibodies towards the endotoxins tested is considerably greater, in most cases, than that of the GX-9 antibodies.

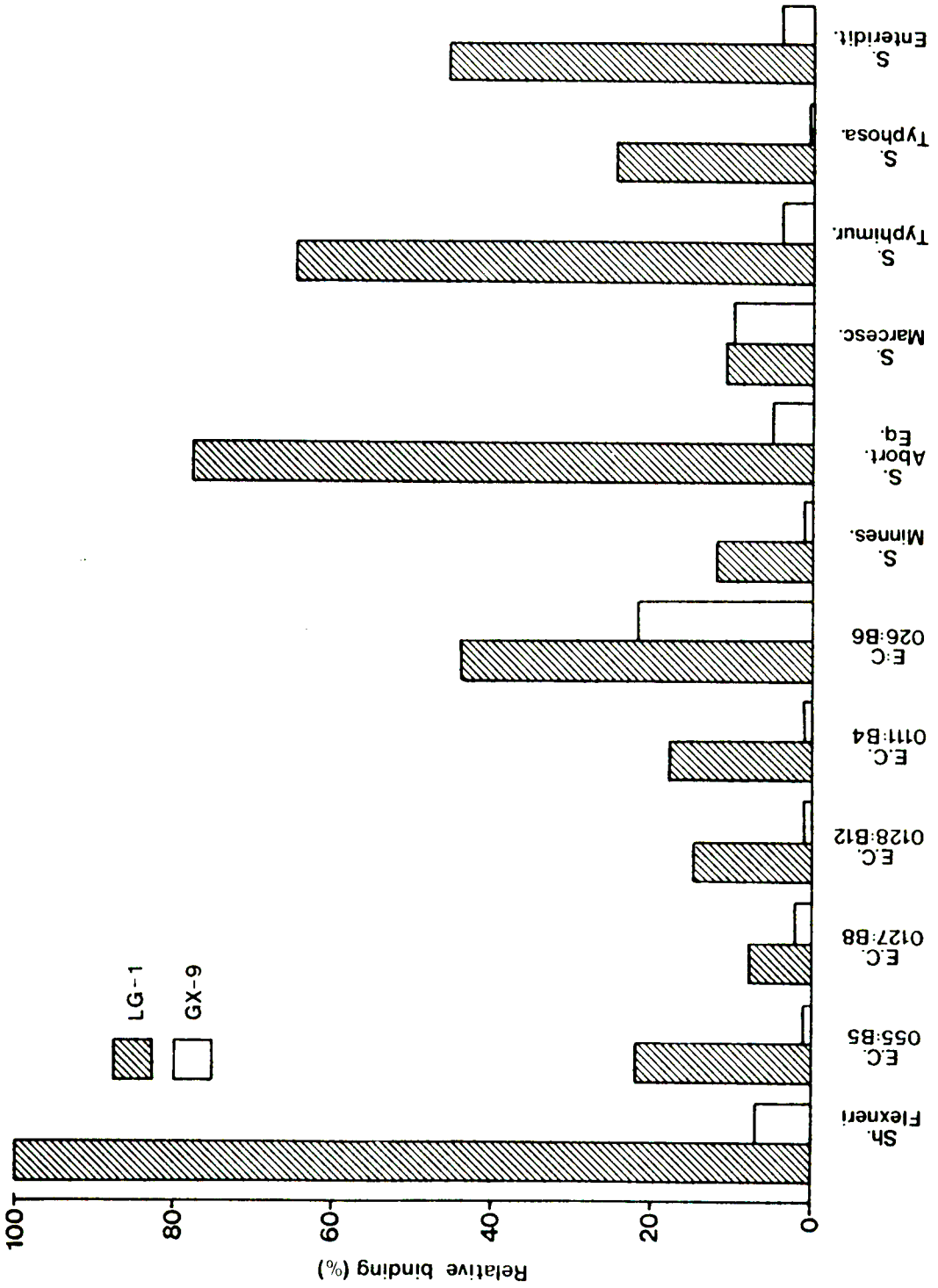

Figure 17. Comparison of LG-1 with an anti-pseudomonas immunoglobulin, GX-9.

4.9 PRESENCE OF CROSS-REACTING AND "SPECIFIC" ANTIBODIES

Differential absorption experiments show that the LG-1 anti-LPS antibodies are a mixture of cross reacting as well as specific antibodies. This is illustrated by the following examples:

LG-1 gamma globulin was first incubated with endotoxin prepared from Shigella flexneri and then assayed on plates which had been coated with all 12 endotoxins. The colour developed in the ELISA procedure was found to be only 60% of that of the untreated control i.e. of the colour developed by LG-1 antibodies binding all 12 endotoxins, 40% was reduced by absorption by Shigella flexneri endotoxin. LG-1 was also incubated with all the other endotoxins at one time, excluding Shigella flexneri endotoxin. In this case the colour developed was 30,7% of the untreated control. This 30,7% is the colour produced by those antibodies reacting specifically with Shigella flexneri endotoxin and not with any of the other endotoxins being tested. The remaining antibodies (9,3%) were cross-reactive.

These results were confirmed by those obtained from assays carried out on plates coated only with Shigella flexneri endotoxin. When Shigella flexneri endotoxin was incubated with LG-1 prior to assay, the activity was reduced to 6,1% of the untreated control. However, when LG-1 was pre-incubated with the other 11 endotoxins minus Shigella flexneri 91,7% of the activity was retained. Antibodies which bound to only Shigella flexneri endotoxin were not absorbed and these could bind to the Shigella flexneri endotoxin coating the plates. Thus a large proportion of antibodies binding to Shigella flexneri endotoxin were mainly specific, however some cross-reactivity is also seen (see Figure 18). Comparable results were obtained with E.coli 026:B6 endotoxin. As shown in Figure 18, 21,0% of the total antibodies absorb E.coli 026:B6 endotoxin; of these however, approximately half are specific and half cross-reacting.

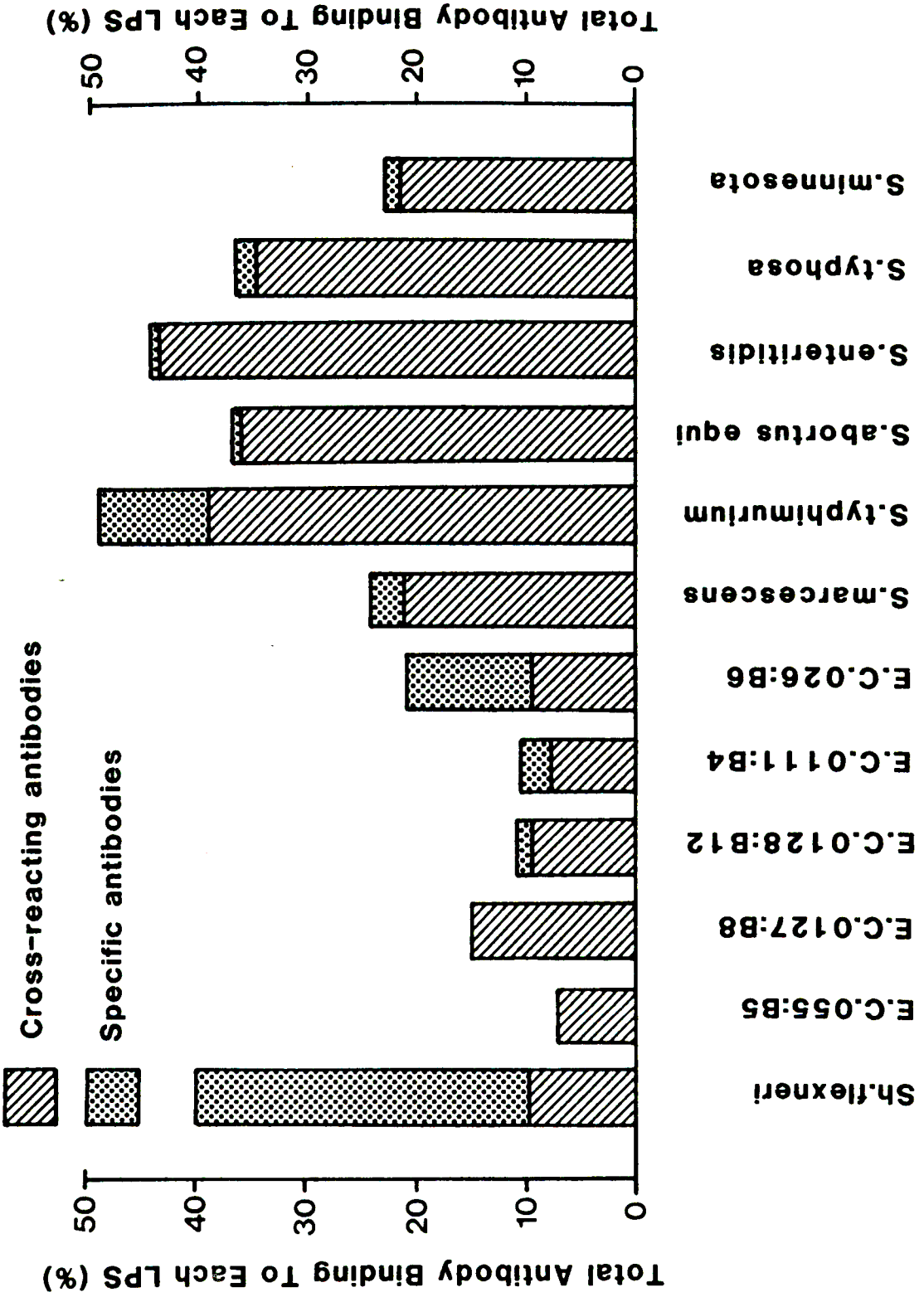

Figure 18. The specificities of LG-1 antibodies to endotoxins prepared from 12 bacterial species and strains.

48,86% of the total antibodies were found to bind to Salmonella typhimurium endotoxin. When LG-1 was incubated with the other 11 endotoxins omitting S.typhimurium, the colour developed on the 12 endotoxin plates was reduced to only 10,67% of the untreated control. Similarly, when this preincubated sample was assayed on plates coated only with S.typhimurium endotoxin, the activity was reduced to 10,5% of the untreated control. Apparently most of the antibodies in LG-1 which react with S.typhimurium endotoxin also bind to the other endotoxins added to the incubation mixture and therefore have been absorbed out, leaving the relatively few specific antibodies, which bind to the S.typhimurium endotoxin coating the plates. From these experiments it was concluded that the antibodies binding to S.typhimurium are largely cross-reactive (78,16%) with, however, some specific antibodies (21,84%) being present. From Figure 18 it can be seen that the antibodies which bind the endotoxins of all the other salmonella species tested (S.abortus equi, S.enteritidis, S.typhosa, S.minnesota) are also largely cross-reactive.

LG-1 antibodies to endotoxins prepared from Serratia marcescens, E.coli 0128:B12 and E.coli 0111:B4 are also mainly cross-reacting. Endotoxins from E.coli 055:B5 and E.coli 0127:B8 are bound only by cross-reacting antibodies. For example, 6,94% of the total antibodies bind E.coli 055:B5 endotoxin. When LG-1 was incubated with the other 11 endotoxins except E.coli 055:B5 endotoxin, the activity on the 12 endotoxin plate was reduced to zero, i.e., there are no antibodies which exclusively bind E.coli 055:B5 endotoxin.

The specificities of all 12 endotoxins tested are summarized in Figure 18 and table 8.

Table 8

Summary of the specificities of the LG-1 antibodies

	Endotoxin species	LG-1 Antibodies
i)	Shigella flexneri	Mainly specific and few cross-reacting
ii)	E.coli 026:B6	Specific and cross-reacting in approximately equal proportion
iii)	Salmonella typhimurium Salmonella abortus equi Salmonella enteritidis Salmonella typhosa Salmonella minnesota Serratia marcescens E.coli 0128:B12 E.coli 0111:B4	Mainly cross-reacting and few specific
iv)	E.coli 055:B5 E.coli 0127:B8	Cross-reacting only

CHAPTER 5

DISCUSSION AND CONCLUSIONS

5.1 DISCUSSION OF RESULTS

In recent years antibody therapy for the treatment of Gram-negative bacteraemia and endotoxaemia has become an experimental adjunct to conventional antibiotic treatment in a very few centres. The principal limiting factor in the past has been the problem of accumulating sufficiently large stocks of human antiendotoxin antibodies. Braude's group used antibodies, raised by vaccination of healthy volunteers with heat-killed Gram-negative bacterial mutants for their highly succesful clinical trials[13-15]. Large stocks of antibodies have not yet however been produced in this manner since immunization with the endotoxin "core" has negative side effects[17].

This problem was overcome in a simple way by screening units of blood donated to a blood bank in order to identify those units which have a high concentration of naturally occurring antiendotoxin antibodies. The high-titre plasma units are pooled and endotoxin specific gamma globulin is prepared from them (as was the case with Lot LG-1), or alternatively individual units may be freeze-dried. The ELISA technique used to measure concentrations of antiendotoxin IgG in the blood samples has proven to be highly specific for these antibodies. Furthermore this procedure could be used by most blood banks since it is relatively inexpensive and does not necessarily require sophisticated equipment.

Individual serum samples, found by ELISA to be high in antiendotoxin IgG, were each seen to have their own characteristic specificities towards different endotoxins. When many high-titre serum samples were pooled and fractionated, the gamma globulin product (i.e. LG-1) was

found to have the ability to bind to a range of endotoxins as shown in Figure 15. This is in contrast to immunoglobulins raised against specific endotoxins; for example, the Wellcome Laboratory GX-9 anti-pseudomonas immunoglobulin preparation did not show significant binding capacity towards the range of endotoxins tested in this study (see Figure 17). The LG-1 gamma globulin also had a far greater capacity to bind endotoxins compared to standard globulin preparations manufactured by the National Blood Fractionation Centre, Pinetown (see Figure 16).

The concentrations of endotoxin-specific antibodies in the blood of different South African population groups has been examined. African donors were found to have a greater incidence of high-titre antiendotoxin antibodies compared to White donors[234]. This may be related to Pudifin and Duursma's findings of higher levels of circulating immune complexes in Black blood donors than in White or Indian donors[236]. African women were, in turn, found to have almost twice the frequency of high titre serum as African men[234]. However, no significant differences in serum antiendotoxin antibody levels were established between Indian male and female hospital patients sampled in this study. Further studies may be necessary to fully understand racial as well as sex differences in antiendotoxin antibody titres.

The differential absorption experiments carried out in this study clearly showed that the LG-1 antiendotoxin gamma globulin is a mixture of cross-reacting as well as specific antibodies. Antibodies which bind to Shigella flexneri endotoxin were found to be largely specific. Those antibodies reacting with E.coli 026:B6 endotoxin were found to be specific and cross-reacting in approximately equal proportions. Antibodies to the salmonella species, Serratia marcescens and remaining E.coli species tested were in the main non-specific. The relatively non-specific nature of the LG-1 antibodies is of advantage in that the preparation can be used to treat endotoxaemia resulting from a range of different endotoxins i.e. it does not act exclusively

with only one or two bacterial strains. Differential absorption experiments had also been carried out on selected high-titre serum samples[234]. A mixture of cross-reacting and specific antiendotoxin antibodies were also found in these individual serum samples.

The "natural" antiendotoxin antibodies present in the high-titre blood samples are presumed to be raised by the donors as a result of previous subclinical infections or due to autoimmunization from occasional intestinal bacteria or endotoxins entering the circulation. One would therefore expect some correlation between the specificities of the LG-1 antibodies and the frequency of different Gram-negative bacteria in the environment. An attempt was made to relate the relative binding activities of the LG-1 antibodies to the reported incidence of various Gram-negative bacteria in blood cultures taken from hospital patients in the area covered by the Natal Blood Transfusion Service.

Figure 19 relates the activity of the LG-1 antibodies (as determined by the relative binding capacity towards the various endotoxins studied) to the incidence of these Gram-negative organisms in blood cultures taken from patients at the King Edward VIII Hospital, Durban, during 1981[96].

The specificities of the LG-1 antibodies towards endotoxins from various Gram-negative bacteria do not in most cases reflect the incidence of these organisms in the blood cultures. For example, LG-1 is high in Shigella flexneri binding antibodies but the incidence in the blood cultures of this species is virtually nil. The incidence of S.typhosa in blood cultures is high; however the binding capacity of LG-1 antibodies towards S.typhosa is relatively low. In contrast, antibodies are abundant towards most of the other salmonella species studied (e.g., S.typhimurium, S.abortus equi, S.enteritidis); however these species are far less commonly encountered in blood cultures. In the case of E.coli and Pseudomonas there appears to be some

correlation between antibody activity and their incidence in blood cultures.

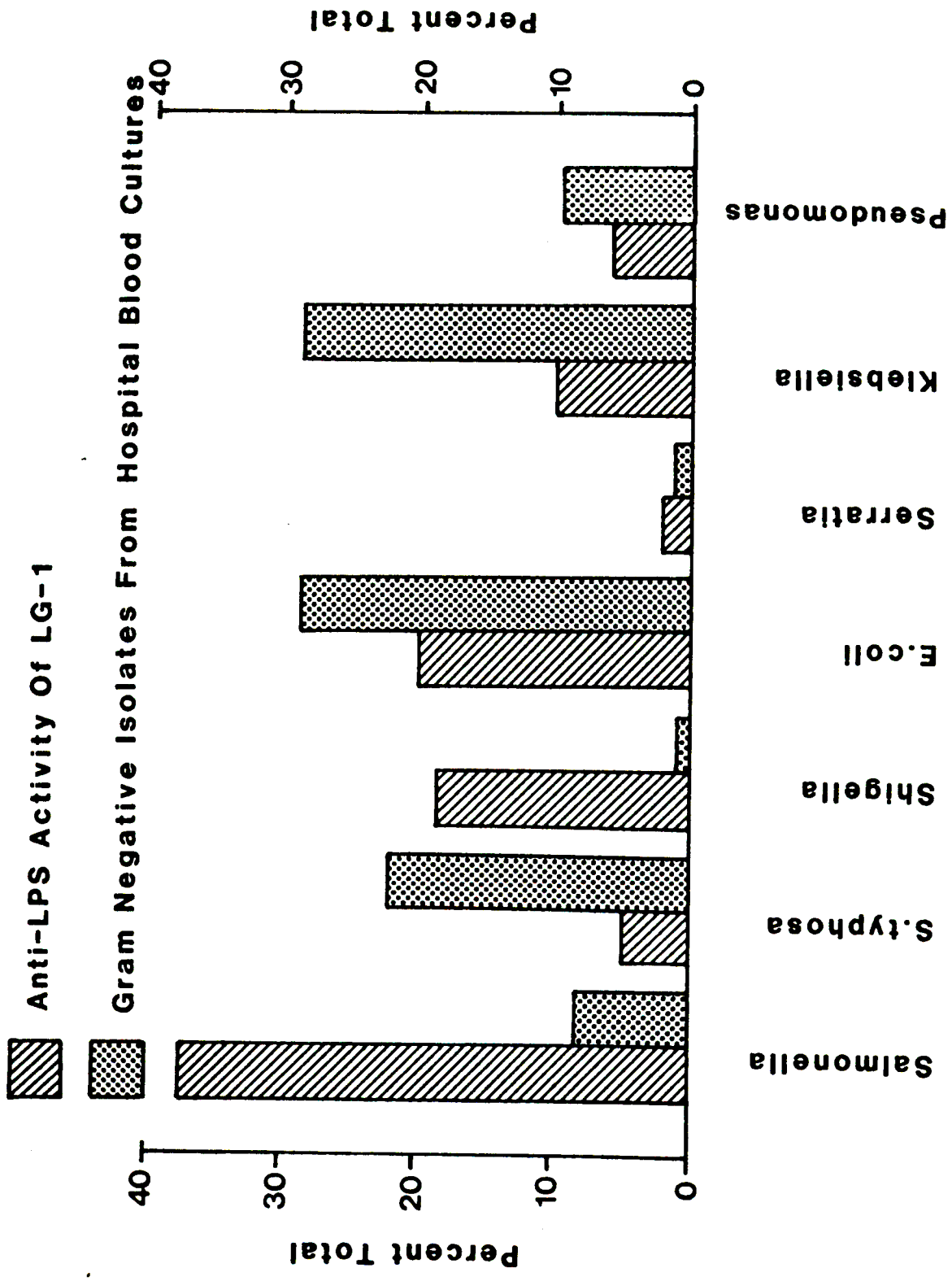

Figure 19. Comparison of LG-1 activity and the frequency of Gram-negative isolates from hospital blood cultures

Shigella is a common cause of intestinal infection but rarely gives rise to bacteraemia; the anti-shigella antibodies reported in this study may be important in restricting the growth of this species in the blood stream and preventing bacteraemia. In a study reported in 1960, Roantree and Rantz examined the heat-labile bactericidal effect of human serum[237]. Almost all the sera tested by them showed the ability to kill Shigella dysenteriae at the rate of 1×10^7 to $1,5 \times 10^8$ organisms/ml of serum in two hours. They also found that a high proportion of the shigella and klebsiella strains tested were sensitive to killing by serum, in contrast to a higher percentage of resistant salmonella strains. There appears to be a limitation to the numbers of strains capable of causing bacteraemia and one of the limiting factors maybe the amount and specificity of the antiendotoxin antibodies present in the host's serum.

Further studies are needed for a deeper understanding of the antiendotoxin antibody specificity. For instance, there may be a high correlation between stool isolates and specific antiendotoxin antibody concentrations.

Gaffin has suggested that the toxic effects of endotoxin may be reduced by specific antibodies by three mechanisms[238]: (a) antibody may bind to the active site of the endotoxin molecule (or close to it) thereby blocking its toxic actions (b) the complex formed between the antibody and endotoxin may be removed from the circulation much faster than endotoxin alone, i.e. the antibody may have an opsonizing effect (c) the binding of antibody to the bacteria activates complement which, in turn, damages the surface membranes of the organisms, leading to their increased permeability, swelling and lysis. In vitro, treatment of Klebsiella pneumoniae with antiendotoxin antiserum caused the cells to agglutinate and form a sticky mass[239]. This required the presence of complement. Electron microscopy showed that the surface membranes of the Klebsiella had

been damaged. Thus, the antiserum appears not only to bind to the endotoxins but also leads to the rapid destruction of Gram-negative bacterial cells[240].

In an important study Vorster and coworkers found that mice treated with LG-1 antibodies prior to inoculation with Pseudomonas aeruginosa were significantly protected against morbidity and mortality as compared with saline treated controls[241]. LG-1 reduced the mortality from 75% to 8% and the morbidity index from 58 to 16.

LG-1, when given prophylactically, was also shown to reduce the abortifacient effect of low doses of E.coli on pregnant rats[242]. The mean weight of the foetuses of the LG-1 treated rats was 1,132g±,0,12g compared to a mean weight of 0,581g±0,3g for the foetuses of the control rats ($p<0,005$).

Shock was induced in rabbits by a 60 minute occlusion of the superior mesenteric artery. Animals which had been treated two days prior to shock induction with antiendotoxin antibodies had a greater rate of survival (7/8; 87,5%) 10 days post shock compared to control animals (2/12; 16,7%)[243].

In view of these successful animal studies Gaffin and coworkers have undertaken numerous clinical studies to test the effectiveness of antiendotoxin immunotherapy in the management of endotoxaemia of different origins. The results of some of these studies are summarized below.

5.2 THE USE OF ANTIENDOTOXIN ANTIBODIES IN THE TREATMENT OF SEPTIC SHOCK IN HUMANS

Twenty-two consecutive patients who were in septic shock or imminent septic shock were treated with antiendotoxin antibodies[244]. The antibodies were administered either intramuscularly in the form of

LG-1 or intravenously in the form of an anti-LPS antibody-rich freeze-dried plasma, FDP, both provided by the Natal Blood Transfusion Service, Pinetown. The patients included in this study had a variety of primary conditions including severe peritonitis and septic shock due to typhoid perforation of the bowel, Hodgkin's disease complicated by sepsis and shock lung and septic shock following pyelonephritis in pregnancy. Twenty of these patients showed complete or temporary improvement in clinical signs. Of the two patients who did not respond at all to antiendotoxin antibody administration one was a child with complete bone marrow failure and severe sepsis and the other was a patient who had brain trauma and it is suggested that his condition might not have been due to septic shock but to the central nervous system damage.

In keeping with these preliminary results are those reported in a separate study in which anti-LPS antibody-rich freeze-dried plasma was used to treat patients in septic shock of obstetrical and gynaecological origin[245]. The patients included in this study were in shock mainly as a result of septic abortions, puerperal sepsis, pyelonephritis in pregnancy or pelvic abscesses. Significant in this study were the stringent criteria used for their definition of shock. Patients were included in this study if they had

(a) pyrexia greater than 38,5°C
(b) an obvious septic focus and
(c) a systolic blood pressure $\leqslant$ 80 mm Hg after resuscitation to a central venous pressure $\geqslant$ 6 cm H_2O.

The control patients received conventional therapy which included replacement of plasma and blood volume, correction of electrolyte and acid base imbalances and the use of antibiotics.

Patients in the study group received similar treatment as well as the intravenous administration of the antibody rich reconstituted freeze-dried plasma. In this Anti-LPS treated group, only 1/14 (7,1%)

patients died while of the control patients, 9/19 (47,4%) died. They found that the antiendotoxin antibody treatment caused the mean arterial pressure (MAP) to rise 24,0 mm Hg from 45,11±7,36 mm Hg to 69,1±9,07 ($t = 6,87$; $p < 0,001$) within a period of 75 minutes from administration. Significantly the development of complications due to septic shock was greatly reduced in the experimental group of patients. In addition the survivors in the control group had a much longer stay in hospital of 28,1 days while those who received antibody therapy had a mean stay of only 14,2 days ($p < 0,05$). It is hoped that the future widespread use of antiendotoxin antibody therapy in the management of septic shock will decrease the overall mortality rate.

In addition to the human antibody preparations, antiendotoxin hyperimmune equine serum has also been produced by vaccination of ponies[246]. The hyperimmune serum has been used in the veterinary field as well as in studies on the treatment of pseudomonas keratitis in rabbits and the treatment of radiation sickness in mice. These studies are summarized below.

5.3 THE USE OF ANTIENDOTOXIN HYPERIMMUNE EQUINE SERUM

5.3.1 Veterinary applications

5.3.1.1 Septicaemia in horses

Hyperimmune equine serum was used successfully to treat the endotoxin mediated diseases colitis X and peritonitis in horses[246]. The antibody rich serum was also effectively used during a gastroenteritis epidemic among foals. Of the 21 foals which received hyperimmune serum (i.v.; 250 ml serum per foal) all completely recovered within 48 hours and subsequently grew normally. 43 foals received the conventional therapy; two died and the remainder needed up to 10 days to recover. Many of these foals subsequently showed stunted growth.

5.3.1.2 Surface infections in horses

The hyperimmune serum was also used topically to successfully treat two mares with intrauterine klebsiella infections[246]. The infections had previously persisted for 12 months and had not responded to prolonged antibiotic therapy.

5.3.1.3 Surgery prophylaxis

Endotoxaemia and death frequently occur following abdominal surgery in horses. Hyperimmune serum is now routinely used prior to surgery on horses and this is reported by veterinary practitioners to have dramatically improved the post-operative mortality and morbidity rates of these horses[246].

5.3.1.4 Prophylaxis in newborn foals

Newborn foals have permeable intestinal walls thus allowing antibodies present in colostrum to enter the blood stream[246]. If, however, the colostrum does not have sufficient antibodies or the foals receive colostrum late then the chance of developing septicaemia increases. Newborn foals were therefore fed per os 250 ml hyperimmune antiendotoxin serum within a few hours of birth in an attempt to supplement the colostrum. Only 2/72 foals which received the hyperimmune serum showed any ill effects while 51/156 of the control animals developed gastroenteritis.

5.3.2 The treatment of pseudomonas keratitis in rabbits

Antiendotoxin hyperimmune equine serum has also been found to be effective in treating corneal pseudomonas infections in rabbits and guinea pigs[247]. The corneas of rabbits were infected with Pseudomonas resulting in scarring and opacity. The eyes were treated by lavage with hyperimmune serum or saline at a rate of 40

drops/minute, for 5 minutes, three times a day for 8 days. Eyes treated with hyperimmune serum improved significantly; 13/15 (86,7%) compared to the saline treated controls 4/17 (23,5%).

5.3.3 The treatment of radiation sickness in mice

Excess ionizing ratiation kills cells lining the gut wall increasing its permeability to bacteria and toxins[243]. These then "leak out" of the gut into the circulation. In addition, excess ionizing radiation has an immunosuppressive effect which further reduces the ability of irradiated animals to counter the effects of invading bacteria and endotoxin. Mice were X-irradiated with 630 rads and 6 days later were treated with 0,1 ml antiendotoxin hyperimmune equine serum (i.p.) or saline. The hyperimmune serum reduced mortality significantly from 71% to 41,1%, compared to controls.

5.4 CONCLUSIONS

From this brief review of the studies of Gaffin and coworkers it can be seen that the potentials for the use of antiendotoxin antibodies are many and varied.

In conclusion, the human antiendotoxin preparations produced by the method of Gaffin and coworkers have a number of advantages which are summarized below:

(a) The preparations contain a mixture of antibodies capable of binding endotoxins from a wide range of Gram-negative bacteria[235,240,248].

(b) Their preparation does not involve any immunization procedures. Large stocks of antibodies can therefore be accumulated since there is no risk to human volunteers.

(c) They are relatively inexpensive to produce and therefore could be available for widespread use.

Studies, both on animals and humans have shown the antibodies to be effective in the treatment and prevention of many of the toxic effects of endotoxin and in time to come the antiendotoxin gamma globulin may be a routine adjunct to conventional therapy in Gram-negative septicaemia.

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LIST OF APPENDICES

Ann Clin Biochem 1982; **19**: 191–194

An ELISA procedure for detecting human anti-endotoxin antibodies in serum

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SUMMARY We report an ELISA method suitable for the large-scale screening of blood bank stores to identify those blood units containing high concentrations of antiendotoxin antibodies. In Natal, 8·3% of total units collected had antiendotoxin antibodies at concentrations greater than 40 μg/ml, values that may be therapeutically useful. We found that one technician could screen enough samples per year to produce 800 litres of such high-titre plasma.

Introduction

The importance of endotoxin in contributing to mortality and morbidity in Gram-negative sepsis and non-septic shock is increasingly recognised.[1 3] However, conventional antibiotic therapy is usually aimed at merely killing bacteria and not at reducing the amount of the very toxic endotoxin present in the blood of bacteraemic and endotoxic patients. This may account for the approximately 50% mortality in Gram-negative bacteraemia. The use of specific antibodies directed against a range of endotoxins would appear to be a possible approach.

Both laboratory animals and humans were protected, sometimes dramatically,[4] against bacteraemia or endotoxaemia by active immunisation against endotoxins or by passive antibody therapy.[5 9] Mainly logistic difficulties prevent the acquisition of large stocks of human endotoxin specific antibodies for large-scale clinical testing. However, we found in Haifa that screening the stocks of blood units present in a blood bank provided a yield of approximately 7% of units containing clinically useful high-titre antiendotoxin antibody-rich plasma.[5] This human plasma was sufficiently antibody-rich to protect cats from a lethal endotoxic shock due to 'irreversible' haemorrhage.[5] However, the screening procedure in these studies was an inefficient and time-consuming immunoprecipitin procedure. Ito and colleagues[10] described an enzyme-linked immunosorbent assay, ELISA,[11] for antibodies to endotoxin.

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We report on a modified procedure that is rapid and specific and suitable for large-scale automated procedures.

Material and methods

Saline solutions and water were sterile and pyrogen free (Sabax Laboratories, Johannesburg). Human serum samples were those obtained along with blood units donated by volunteers in the Natal Province using the facilities of the Natal Blood Transfusion Service. Samples were usually tested within three days of collection. Before use the serum was centrifuged for 5 minutes at 16 000 rpm and then diluted 1:50 with 0·9% NaCl. Endotoxins (Difco) were obtained from 12 bacterial strains and species (*E. coli* 055: B5; *E coli* 0127: B8; *E. coli* 0128: B12; *E. coli* 026: B6; *E. coli* 0111: B4; *Shig. flexneri*; *S. minnesota*; *S. abortus equii*; *S. marcescens*; *S. typhosa*; *S. typhimurium*; *S. enteritidis*). They were prepared as stock solutions with water (1 mg/ml) and stored in small volumes frozen until used. The general ELISA microplate method used here has been described previously for alpha fetoprotein and other applications.[12 13] Microtitre plates (Dynatech M129B) were coated with a mixture of endotoxins for 2 hours at room temperature. Conjugate consisting of antibody to human IgG and horseradish peroxidase was made according to the method of Wilson and Nakane.[14]

Results

ENDOTOXIN COATING OF MICROTITRE PLATES

When a mixture of endotoxins at different concentrations was added to the wells of the microtitre

plate for coating, the intensity of the final colour depended on the concentration of endotoxin placed into the wells.

For three different serum samples at endotoxin concentrations less than 10 μg/ml the colour intensity was low, but at 10 μg/ml and higher, the optical density increased substantially (Fig. 1). For reasons of economy, all further studies were carried out at an endotoxin concentration of 10 μg/ml, a value that provided appropriate colour intensities.

CALIBRATION

Serum sample number 104 was found to have an anti-endotoxin antibody concentration of 44·4 μg/ml according to an immunoprecipitin technique.[5] This sample was diluted with 0·1M NaCl and tested by ELISA at various concentrations, as shown in Figure 2. For rapid screening purposes the optical density curve was divided into four regions for easy visual subjective evaluation of 0, +, ++, and +++. These designations corresponded to anti-endotoxin antibody concentration ranges of 0–2·5 μg/ml, 2·5–5 μg/ml, 5–40 μg/ml, and greater than 40 μg/ml, respectively.

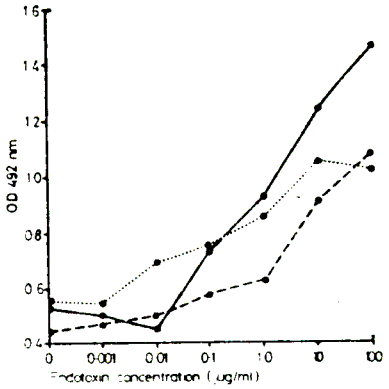

Fig. 1 *Effect of endotoxin concentration during the coating of microtitre plated on ELISA colour development in three serum samples.*

SPECIFICITY OF ELISA FOR ENDOTOXINS

When high-titre serum samples according to ELISA were preincubated with a mixture of endotoxins for 1 hour at 37°C and then assayed, the ELISA was negative. Apparently the antiendotoxin antibodies present bound to the added endotoxins and so were unavailable to bind to the solid-phase endotoxin.

DISTRIBUTION OF ANTIBODY CONCENTRATIONS

Figure 3 shows the distribution of antiendotoxin antibody titres among 1051 blood units. The largest group (42·2%) tested had zero to 2·5 μg/ml. The next largest (31·7%) had 2·5–5 μg/ml. A smaller group tested (16·8%) had 5–40 μg/ml and the smallest group had more than 40 μg/ml (8·3%), a value previously found to be therapeutically useful in cats.[5]

SPECIFICITY OF HIGH-TITRE SERA FOR VARIOUS ENDOTOXINS

Some plates were coated with pure endotoxins obtained from any one of the 12 different bacterial species and strains. Samples tested by ELISA on these plates yielded characteristic reactivities, shown in Figure 4. The height of each bar represents the antibody concentration measured in 12 selected

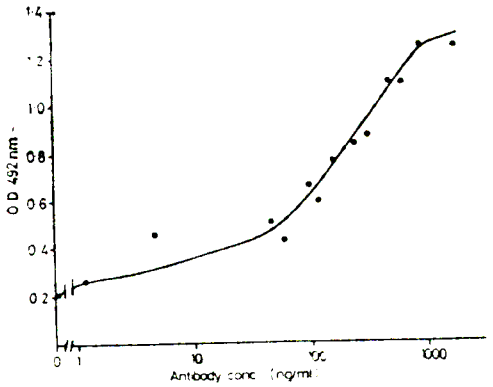

Fig. 2 *Calibration of ELISA.*

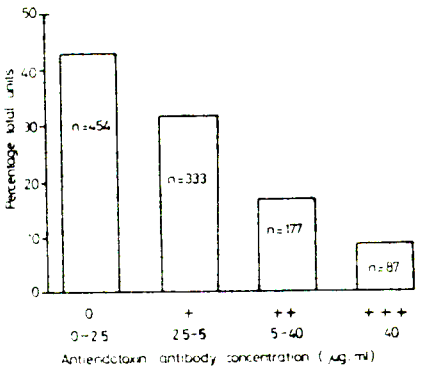

Fig. 3 *Distribution of antiendotoxin antibody concentrations in 1051 plasma samples from blood bank stocks.*

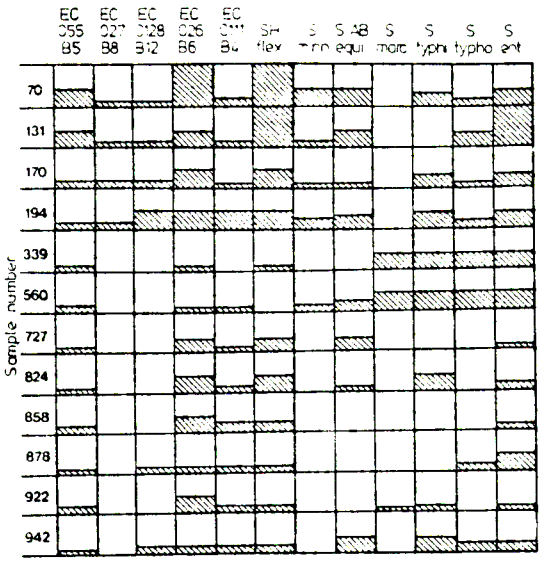

Fig. 4 *Specificities of antiendotoxin antibodies toward entotoxins obtained from 12 different bacterial strains and species in 12 high-titre (>40 μg/ml) plasma samples. Most samples reacted strongly with* S. typhmurium, S. enteritidis, *and* Sh. flexneri *but poorly or not at all with* S. marcesens, E. coli *0111:B4*, E. coli *027:B8*, *and* S. minnesota.

serum samples. Some high-titre samples reacted strongly against certain strains of *E. coli* endotoxin (eg, 026: B6) but not against others (0111:B4). They reacted more or less strongly against most of the species tested but rarely against *S. marcesens* or *E. coli* 0111:B4, each serum sample having its own characteristic specificities.

Discussion

A number of controlled studies have shown the significant therapeutic benefit of passively transferring human antibodies specific to endotoxins to appropriate patients with confirmed Gram-negative bacteraemia or endotoxaemia. Mortality was reduced by 50% or even considerably more.[4 9] In most of these studies, however, the antibodies were produced by actively immunising volunteers, a process requiring the utmost care and control since immunisation is done with endotoxins, endotoxin mutants, or a Gram-negative bacterial cell wall extract, a mixture including endotoxins. Endotoxin is toxic in humans at a concentration of 10^{-9} g/ml or about 10^{-12} M and thus is at least 1000 times as toxic as the nerve toxin tetrodotoxin found in puffer fish (Tetraodontidae), which is no longer toxic at 10^{-9}M.

Major difficulties in producing large volumes of human antiendotoxin antibodies are the legal, practical, administrative, and ethical problems in dealing with large numbers of subjects using a potentially hazardous immunogen. These problems can be avoided by simply screening the blood units in the blood bank by the above ELISA procedure and reserving those high-titre units found for treating endotoxaemia or Gram-negative sepsis. In our laboratory, one technician working with automatic pipetting and optical reading equipment can screen by means of the ELISA described here some 200 serum samples per day or 50 000 per year. With an effective recovery of 8% (Fig. 3) this could provide 4000 units or approximately 800 litres of high-titre plasma per year. A different published ELISA procedure using tubes rather than microtitre plates and much greater amounts of endotoxin for coating purposes may be satisfactory in a laboratory but would be more costly and is not well suited for large-scale screening.[10] An extrapolation from animal studies suggests that 3 ml/kg of high-titre (40 μg/ml) antiserum may be therapeutically useful.[5 8] Thus the minimum annual yield of one technician could produce enough high-titre plasma to treat *ca.* 88 800 3-kg neonates or 3800 70-kg adults. A different study on humans suggests that substantially less hyperimmune serum would be required, and hence a much greater number of clinical cases could be treated.[4]

Antibodies found by this screening procedure bind with endotoxins obtained from a few different Gram-negative bacteria, each serum sample containing antibodies with its own characteristic specificities. By pooling a large number of such high-titre units, the final product obtained had a very wide range of reactivities. Such pooled plasma contained antibodies that could bind to Gram-negative bacteria and together with complement could lyse them, even those outside the listed range including *Klebsiella pneumoniae*.[15] We have previously shown that such high-titre serum found by screening in a blood bank is effective therapeutically in treating cats in a lethal endotoxaemia secondary to a haemorrhagic shock.[5] Presumably human serum would be at least as effective in humans, and controlled double-blind clinical trials of this material are now in progress. Thus blood bank screening can provide an immediate source of anti-endotoxin antibodies.

We are grateful to Mrs Maureen Rome for her excellent technical work.

The research was supported by South African Medical Research Grant n. 4201-68.

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Accepted for publication 17 September 1981.

Antiendotoxin Antibody Specificities among African and Caucasian Blood Donors in Natal

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Endotoxin, a lipopolysaccharide located on the outer surface of gram-negative bacteria, is believed to be the major causative factor in the 50% mortality in gram-negative sepsis, which results in *c.* 100 000 deaths per year in the United States. Under certain conditions, endotoxin may enter the systemic circulation from the intestine and cause additional shock and mortality. We have shown that an otherwise lethal endotoxaemia, induced by haemorrhage and resulting from intestinal endotoxin invasion, can be reversed by the use of human antiendotoxin antibodies obtained by screening random blood units in a blood bank [Gaffin *et al.* (1981). *J. surg. Res.* **31**, 18]. The immunoprecipitin procedure employed as a screening test was very slow and inefficient. We have now developed a rapid Enzyme Linked Immunosorbent Assay (ELISA) for detecting the presence of human anti-endotoxin antibodies. Using this procedure some 100 000 blood units may be screened in triplicate per year. In Natal, blood units containing high concentrations of specific antibodies ($> 40\ \mu g/cm^3$), were found with a frequency of 3.6% among white donors ($n = 1658$) and 10.3% among African donors ($n = 2482$, $P < 0.01$). The white male and female donors showed no significant differences, but the African women had almost twice the frequency of high titre serum as the African men (14.43% versus 7.68%, $P < 0.01$).

Differential absorption experiments were carried out on selected serum samples. Sera were preincubated with one or several pure endotoxins prior to determining quantitatively the antiendotoxin antibody activity remaining in the absorbed sera on ELISA plates coated with one or a mixture of endotoxins. Serum samples were found to contain mixtures of antiendotoxin antibodies. Some of these antibodies cross-reacted with many, but not all, endotoxins used, while other antibodies appeared to bind only one endotoxin.

Antibiotic Effect of Human and Equine Antiendotoxin Antibody-rich Serum on *Klebsiella pneumoniae*

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The administration of endotoxin-specific antibodies improves survival in gram-negative sepsis in a variety of animals and humans. We have investigated the bactericidal effects of human and equine antiendotoxin antibody-rich serum samples. Human high titre serum ($> 40\ \mu g/cm^3$) was obtained by screening blood units in a blood bank by an Enzyme Linked Immunosorbent Assay (ELISA) [Gaffin *et al.* (1982). *Ann. clin. Biochem.* in press]. Equine serum was obtained by appropriate immunization of horses. Serum samples were placed into wells of agar plates 'seeded' with *Klebsiella pneumoniae*. Those wells containing high titre serum inhibited growth around them.

Treating *K. pneumoniae in vitro* with antiserum caused the cells to 'agglutinate' and form a sticky mass. This process required the presence of complement. Electron microscopy showed that surface membranes of *Klebsiella* were affected by the antiserum, leading to an increased permeability, swelling, and lysis. Cell contents released from lysed bacteria apparently caused the remaining membranes and cell components to adhere together as a solid mass. The antiendotoxin antibody-rich serum does more than bind to endotoxins; it leads to the destruction of gram-negative bacterial cells.

108

PRODUCTION AND PROPERTIES OF HUMAN ANTIENDOTOXIN ANTIBODY RICH GAMMA GLOBULIN.S.L. GAFFIN, N.BADSHA, M. IPSCORY, J.BROCK-UTNE,B.VORSTER, J.CONRADIE. Dept. Physiology, Univ. Natal Medical School, Durban, and Natal Blood Transfusion Service, Pinetown. South Africa.

Studies by Braude's group, McCabes' and Jones' have shown the protective effect of human antiendotoxin antibodies on gram negative sepsis and endotoxemia in animals and man. However, such antibodies are not available for general clinical use. We found that simply screening the units of blood in a blood bank can provide substantial amounts of AntiET Ab rich human plasma (Gaffin et al., 1981.J.Surg.Res. 31:18) which protected cats from lethal endotoxemia caused by hemorrhagic shock. An ELISA procedure has been used for larger scale screening (Gaffin et al. 1982.Ann. Clin.Biochem. In Press) and a gamma globulin fraction (Lot LG-1) was prepared. The total protein conc. is 16%, the antiET ab concentration is 1200 ug/ml and the relative reactivities of these Ab's prepared from various bacteria is 100:24.5:10.4: 21.2: 46.8: 21.4: 9.5: 83.2: 13.8: 67.9: 29.3: 45.1: for Sh. flexneri; E coli 055B5: Ec 0127B8: Ec 021B12: Ec 026B6 Ec 0111B4: S. minnesota, S. abortus equi: S. marcescens: S. typhimurium: S. typhosa: S. enteredtis, respectively. This globulin together with complement could lyse and kill a range of gram negative bacteria, including Klebsiella. Electron microscopic studies show that with LG-1 and complement the rod shaped gram negative bacteria swell, become baloon shaped, burst, release their cytoplasm and the membranes form ghosts. Clinical trials using LG-1 are in progress. Supported by SAMRC grant 4201-68 and the Loewenstein Foundation.

48

PROPERTIES OF HUMAN LPS SPECIFIC GAMMA GLOBULIN
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We have produced human LPS specific gamma globulin (Lot LG-1) currently undergoing clinical evaluation. BALB/C mice were inoculated i.p. with 0.1ml of live cultures of Pseudomonas aeruginosa (Type P14) or placebo. LG-1 reduced mortality from 75% to 8% and morbidity index from 58 to 16.
We previously reported that Ab's in LG-1 bound to LPS from a wide range of gram negative bacteria. LG-1 was absorbed with various pure and mixtures of LPS prior to ELISA measurement of remaining Ab activity on microtiter plates coated with one or a mixture of LPS. This compared the relative amounts of various "specific" and cross reacting anti LPS Abs present in LG-1. 76.8% of Abs binding Sh.flexneri were specific, 56.5% to E.coli 026:B6, 33.4% to E.coli 0111:B4, 21.8% to S.typhimurium. The remainders of Anti LPS bindings were "cross reactive". Only 0-15% specific binding was seen in Abs to E.coli 055:B5, 0127:B8, 0128:B12, S.marcescens, S.ab.equii, S.enteretidis, S.typhosa and S.minnesota. Presumably the relative abundance of Abs in LG-1 reflects previous infections. However Ab's binding to Sh.flexneri were most abundant in LG-1, but Shigella isolates were <1% of total. Klebsiella binding Ab's were 10.1% of LG-1 but 29.1% of total isolates.

PROPERTIES OF HUMAN ANTILIPOPOLYSACCHARIDE (ANTI-LPS) SPECIFIC GAMMA GLOBULIN : SPECIFICITY AND PROTECTIVE EFFECTS##

by

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Running Title: ANTI-LPS SPECIFIC GLOBULIN

Supported by grants from SA MRC and Anglo American Corporation

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SUMMARY

Blood donated to the Natal Blood Transfusion Service was screened by an ELISA for Anti-LPS antibodies. Plasma units with high concentrations (> 40 µg/ml) of Anti-LPS IgG were pooled and fractionated to obtain a gamma globulin (Lot LG-1). The binding of LG-1 antibodies to LPS prepared from 14 bacterial species and strains was found to be the highest to LPS from Shigella flexneri, S. abortus equi and S. typhimurium and intermediate with Klebsiella pneumonia, Pseudomonas aeruginosa, S. enteritidis and E. coli 026:B6. Differential absorption experiments showed that LG-1 contained a mixture of specific and cross-reacting antibodies. A large proportion of antibodies binding to Shigella flexneri LPS were mainly specific, while those binding to S. typhimurium and the other Salmonella species tested were largely cross-reactive. There was little correlation between the spectrum of activity of the LG-1 antibodies and the incidence of gram negative bacteria in blood cultures taken from hospital patients in an area covered by the Transfusion Service. Mice treated with LG-1 prior to inoculation with Pseudomonas aeruginosa were significantly protected against morbidity and mortality compared to controls.

INTRODUCTION

It is increasingly recognised that a major component of mortality and morbidity in gram negative septicaemia and nonseptic shock is the lipopolysaccharide, (LPS, endotoxin) released from the surface of gram negative bacteria, or which may enter from the gut where it is always present[1,2]. With conventional antibiotic therapy alone mortality in such cases is approximately 30-50 per cent; in part because the LPS remains toxic even though the bacteria which produced it are dead[3]. The administration of specific anti-endotoxin antibodies has significantly reduced this high mortality and morbidity in animals and man[4,5,6,7,8].

Unfortunately, substantial quantities of human anti-lipopolysaccharide (Anti-LPS) are not presently available for clinical use, in part due to logistic, ethical and legal problems involved in immunizing volunteers with a potentially toxic substance. We partially solved this problem by recognizing that significant numbers of blood units donated to a blood bank contain raised levels of Anti-LPS antibodies[6], developing an ELISA procedure to rapidly screen donated blood, retaining and pooling those units with high concentrations of Anti-LPS[9]. Such human Anti-LPS was protective in animals against septic abortion[10] and in humans against septic shock[7,11]. We report here on the properties of a gamma globulin (Lot LG-1) prepared from such high titre human plasma.

MATERIALS AND METHODS

1. PREPARATION: ANTI-LPS SPECIFIC GAMMA GLOBULIN

An ELISA was used to measure Anti-LPS in all studies reported here[9]. This ELISA had been calibrated by an immunoprecipitin procedure[6]. The ELISA was performed in microtitre plates which had been precoated with a mixture LPSs prepared by the Westphal extraction procedure (SIGMA) from the following bacteria: E. coli 0127:B8, E. coli 0128:B12, E. coli 0111:B4, E. coli 026:B6, S. minnesota, Shigella flexneri, S. abortus equi, S. marcescens, S. typhosa, S. typhimurium and S. enteritidis. Blood donated to the Natal Blood Transfusion Service, Pinetown, South Africa was routinely screened for the presence of Anti-LPS IgG, usually within three days of donation. Those units containing LPS-precipitible antibodies at concentrations greater than 40 µg/ml were stored frozen at -20°C until 50 litres accumulated. This plasma was pooled and fractionated at the National Blood Fractionation Centre according to a standard licensed procedure using Kistler modification of the Cohn method[12] and glass ampules were filled with this gamma globulin labelled Lot LG-1. Before use, Lot LG-1 underwent the normal quality controls applicable to all gamma globulin preparations.

2. RELATIVE ACTIVITY OF LG-1 ANTIBODIES

LG-1 was diluted 1:800 in 0.1M NaCl containing 0.5M Tris adjusted to pH 8,0 and also containing 2% each of sheep and bovine serum. The assay was carried out on microtitre plates coated with only one each of the following endotoxins: E. coli 055:B5; E. coli 0127:B8; E. coli 0128:B12; E. coli 0111:B4; E. coli 026:B6; S. minnesota; Shigella flexneri; S. abortus equi; S. marcescens; S. typhosa; S. typhimurium; S. enteritidis; Klebsiella pneumonia and Pseudomonas aeruginosa. Several wells on every plate were coated with LPS from Shigella flexneri to use as a reference standard.

3. DIFFERENTIAL ABSORPTION EXPERIMENTS

LG-1 was preincubated with a variety of solutions containing LPS and then tested for antibody activities remaining. LG-1 was diluted 1:800 in 0,1M NaCl containing 0.5M Tris adjusted to pH 8,0 and also containing 2% each of sheep and bovine serum. This solution was used in the following incubations below, all carried out at 37°C for 10 minutes. In each case endotoxin was added to a final concentration of 50 µg/ml.

(i) The diluted LG-1 was incubated separately with each LPS prepared from the same bacteria as described above in 2 omitting K. pneumoniae and P. aeruginosa.

(ii) Diluted LG-1 was incubated with a mixture of all twelve endotoxins, each at a concentration of 4,16 µg/ml.

(iii) Diluted LG-1 was incubated with mixtures of eleven of the twelve LPS by omitting each one in turn.

(iv) As controls the diluted LG-1 was incubated with 0,1M acetate buffer, pH 5,0 (the LPS buffer).

The above absorbed LG-1 were centrifuged at 4°C for three minutes at 16 000 x g. They were then assayed for Anti-LPS IgG. The assay was carried out on two types of microtitre plates : those coated with only one of each of the first 12 of the above LPS separately, and those coated with a mixture of all of the first 12 LPS, together.

4. MOUSE PROTECTION STUDIES

Each of twelve Balb C mice matched for weight received either 0,1 ml of 0,15M NaCl or 0,1 ml LG-1 intraperitoneally. One hour later all mice were inoculated with 1,0 ml of a suspension of Pseudomonas aeruginosa type P14 containing 1,1 x 10^9 organisms/ml*.

* A gift of Dr R J Jones, Accident Hospital, Birmingham, U K.

They were placed in separate cages and classified at 2 hourly intervals by an experienced animal handler from 0 = no change, healthy, to + 5 = prostrated, breathing, to + 6=death. Mice surviving 20 hours after administration of Pseudomonas aeruginosa were considered "protected".

RESULTS

The ELISA colour development, and hence the binding, of LG-1 antibodies to lipopolysaccharide prepared from 14 species and strains is shown in Fig. 1. Binding was found to be highest to LPS from Shigella flexneri, S. abortus equi and S. typhimurium, and at intermediate levels with S. enteritidis, K. pneumoniae and P. aeruginosa and E. coli 026:B6. Binding to the other LPS was relatively low.

Differential absorption experiments show that LG-1 Anti-LPS antibodies are a mixture of cross-reacting as well as specific antibodies. This is illustrated (Fig. 2) by the following examples :

LG-1 was first incubated with LPS prepared from Shigella flexneri and then assayed on plates which had been coated with all 12 LPS. The colour developed in the ELISA procedure for this Shigella preincubated sample was found to be only 60% of that of the untreated control, i.e. of the colour developed by all antibodies in LG-1 binding to all 12 endotoxins coating the plate. Forty per cent of the colour was lost by absorption of LG-1 by Shigella flexneri endotoxin. LG-1 was then preincubated with a mixture of the other eleven LPS excluding Shigella flexneri endotoxin. In this case the colour developed was 30,7% of the untreated control. This 30,7% represents colour produced by those antibodies reacting specifically with Shigella flexneri endotoxin and not absorbed out by any of the other endotoxins being tested. The remaining antibodies (9,3%) were cross-reactive.

When LG-1 was incubated with Shigella flexneri and then assayed on plates coated only with Shigella LPS, the activity was reduced to only 6,1% of the untreated control. However, when LG-1 was preincubated with the other 11 LPS still 91,7% of the activity was retained. In this latter case antibodies which bound to only

Shigella flexneri LPS were not absorbed out and these could bind to the Shigella flexneri LPS coating the plates. Thus a large proportion of antibodies binding to Shigella flexneri were mainly specific, but with some cross-reactivity. (See Fig. 2). Comparable results were obtained with E. coli 026:B6 LPS; of these, however, approximately half are specific and half cross-reacting.

A very different set of results was seen with S. typhimurium. 48,86% of the total antibodies were found to bind to Salmonella typhimurium LPS. When LG-1 was incubated with the other 11 LPS, omitting S. typhimurium, the colour developed on the 12 LPS plates was reduced to only 10,67% of the untreated control. Similarly, when this preincubated sample was assayed on plates coated only with S. typhimurium LPS, the activity was reduced to 10,5% of the untreated control. Apparently most of the antibodies in LG-1 which react with S. typhimurium LPS also bind to the other LPS added to the incubation mixture and therefore have been absorbed out, leaving the relatively few specific antibodies, which bind to the S. typhiumurium LPS coating the plates. From these experiments it is concluded that the antibodies binding to S. typhimurium are largely cross-reactive (78,16%), with the balance probably being specific.

From Fig. 2 it can be seen that the antibodies which bind the LPS of all the other Salmonella species tested (S. abortus equi; S. enteritidis; S. typhosa; S. minnesota) are also largely cross-reactive.

LG-1 antibodies to LPS prepared from Serratia marcescens, E. coli 0128:B12 and E. coli 0111:B4 are also mainly cross-reacting. Endotoxins from E. coli 055:B5 and E. coli 0127:B8 are bound only by cross-reacting antibodies. For example 6,9% of the total antibodies bind E. coli 055:B5 LPS. When LG-1 was incubated with the other 11 LPS except E. coli 055:B5 LPS, the activity on the 12 LPS plates was reduced to zero, i.e. there are no antibodies which exclusively bind to E. coli 055:B5 LPS.

The specificities of all 12 LPS tests are summarized in Fig. 2 and Table 1.

MOUSE PROTECTION

There were marked differences between the groups of mice given placebo or LG-1 after inoculation with Pseudomonas culture (P14) (Fig. 3). The morbidity curve of the controls reached half its maximum plateau value within five hours and its maximum plateau value within 24 hours. The LG-1 pretreated mice showed only slight morbidity at 18 hours which then subsided until 40 hours postinoculation. Its final value was only 16 arbitrary units compared to the controls' 58. The first death of the control mice occurred 18 hours after inoculation and 50% had died by 36 hours postinoculation. On the other hand the LG-1 treated mice had a very low mortality (1/12) occurring after 50 hours postinoculation.

DISCUSSION

LG-1 was prepared by the screening of approximately 3000 plasma units resulting in 50 litres of high titre plasma. We previously found that each donor had his own particular group of antibodies which bound various LPS to a different extent - one donor having high levels of antibodies against, say, E. coli 055:B5,another to Sh. flexneri and another to S. typhosa[9]. The final product LG-1 thus contains antibodies to the LPS of a wide range of different gram negative bacteria.

Anti-LPS hyperimmune serum and plasma have been shown to reduce mortality and morbidity in a wide range of gram negative bacterial infections and endotoxaemias in animals and man[4-8]. Because of its beneficial effect in endotoxin and mediated diseases, equine Anti-LPS has become almost a routine therapy in the equine veterinary industry in South Africa[13].

A question remains as to whether there are present in our LG-1, anti-gram negative bacterial antibodies other than those specific to LPS which also contribute to the observed beneficial effect of LG-1 on mortality and morbidity in the infected mice. At present, this possibility cannot be excluded. A controversy persists as to the therapeutic advantage of an IgM rather than an IgG preparation[4]. We and others found an IgG preparation to be therapeutic against: Pseudomonas lethality in mice, endotoxin shock induced by prolonged haemorrhage in cats[6], septic abortion in pregnant rats[10], and infections in mice[14] and humans[15] with large burns. IgG has the benefits of having a substantially longer half-life in circulation and ease of preparation compared to IgM.

An important problem has been how to prepare human Anti-LPS on a substantial scale for human use. For a period of two years a monthly incidence of 6,8 - 7,2% of donated blood had high concentrations (40 > µg/ml) of Anti-LPS IgG. For a blood bank collecting 200 units per day this yield provides 7% x 200/day x 5 days/wk x 50 weeks = 3500 Anti-LPS rich plasma units per year. Our experience

is that the antibodies in approximately six units of such plasma are therapeutic for severe gram negative septic shock in humans[11]. Therefore 480 adult doses can be prepared per year. Due to the long shelf life of gamma globulin, it can be stored well for emergency use. It may be used with fewer licensing problems as a freeze dried plasma. Ultimately, an intravenous form of the gamma globulin should be prepared because of the importance of immediate availability of the antibodies for patients in shock. Inevitably, however, such procedures deplete those protective LPS specific antibodies in the general pool of the low titre gamma globulin.

The reason why certain donors have high levels of Anti-LPS is not known but is expected to be due in part to the donor having had a recent subclinical infection. An attempt was made to relate the relative binding activities of LG-1 antibodies to the reported incidence[16] of various gram negative bacteria in blood cultures in hospital patients in the area in which blood is donated.

It is seen in Fig. 4 that in most cases the incidence of blood cultures is not correlated with the binding of the antibodies to various LPS. For example LG-1 is high in IgG binding to Sh. flexneri but the incidence of Sh. flexneri in blood cultures is virtually nil. The incidence of S. typhosa in blood cultures is high, yet binding of LG-1 antibodies towards S. typhosa LPS is relatively low. In contrast, antibodies abundant towards most of the other Salmonella species; these species, however, are far less commonly encountered in blood cultures. In the case of E. coli and Pseudomonas there was some similarity between antibody activity and their incidence in blood cultures.

Shigella is a common cause of intestinal infection but rarely gives rise to bacteraemia; the anti-shigella antibodies reported here may be important in restricting the growth of this species in the bloodstream and preventing bacteraemia. There appears to be a limitation to the number of strains capable of causing bacteraemia and one of the limiting factors may be the amount and specificity of the Anti-LPS antibodies present in the host's plasma. Further epidemiological studies are needed for a deeper understanding of the Anti-LPS antibody specificity. For instance there may be a high correlation between stool isolates and specific Anti-LPS concentration.

We routinely produce human LPS specific globulin and anti-LPS freeze dried plasma with the facilities of a medium sized blood bank. The Anti-LPS IgG binds to a wide range of gram negative bacteria, was protective against pseudomonas bacteraemia in mice, and was previously found to be protective against E. coli induced abortion in pregnant rats[10]. Such preparations may be valuable adjuncts to conventional antibiotic therapy in septicaemia[4,5,7,11].

ACKNOWLEDGEMENTS

We thank Mr H J Klomfass for his excellent technical assistance and Dr Brian Feery for the Australian blood samples.

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LEGENDS

Figure 1 The Relative Binding Capacities of IgGs present in human LPS specific globulin (LG-1) to lipopolysaccharides prepared from 14 bacterial species and strains.

Figure 2 The Specificities of LG-1 antibodies to LPSs prepared from 12 bacterial species and strains. Except for Sh. flexneri, most showed considerable cross reactivity.

Figure 3 Morbidity and Mortality in Pseudomonas infected mice. Mice received 0.1 cc i.p. of LG-1 (Solid Symbols) or 0.9% NaCl (Open Symbols). One hour later they were infected with Pseudomonas (10^9 org.). Both the Morbidity Index (Circles) and the Mortality (Squares) of LG-1 treated mice were less than those in the controls.

Figure 4 Comparison of LG-1 activity and the frequency of hospital isolates.

Table 1 Cross reactivities of IgGs present in LG-1 Summary.

TABLE 1

	LPS SPECIES	LG-1 ANTIBODIES
i)	Shigella flexneri	Mainly specific and few cross-reacting
ii)	E. coli 026:B6	Specific and cross-reacting in approximately equal proportion
iii)	Salmonella typhimurium	Mainly cross-reacting and few specific
	Salmonella abortus equi	
	Salmonella enteritidits	
	Salmonella typhosa	
	Salmonella minnesota	
	Serratia marcescens	
	E. coli 0128:B12	
	E. coli 0111:B4	
iv)	E. coli 055:B5	Cross-reacting only
	E. coli 0127:B8	

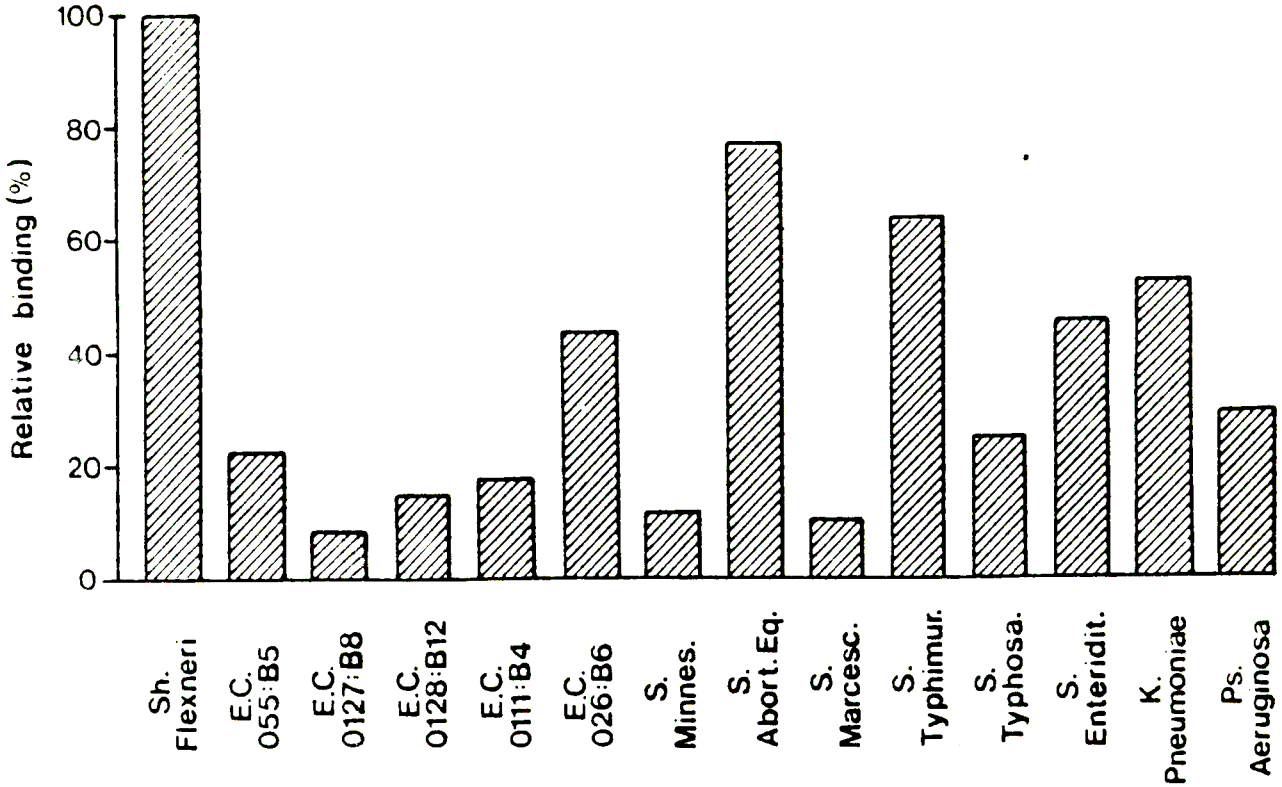
Relative binding (%)
100
80
60
40
20
0
Sh. Flexneri
E.C. 055:B5
E.C. 0127:B8
E.C. 0128:B12
E.C. 0111:B4
E.C. 026:B6
S. Minnes.
S. Abort.Eq.
S. Marcesc.
S. Typhimur.
S. Typhosa.
S. Enteridit.
K. Pneumoniae
Ps. Aeruginosa

Fig. 1

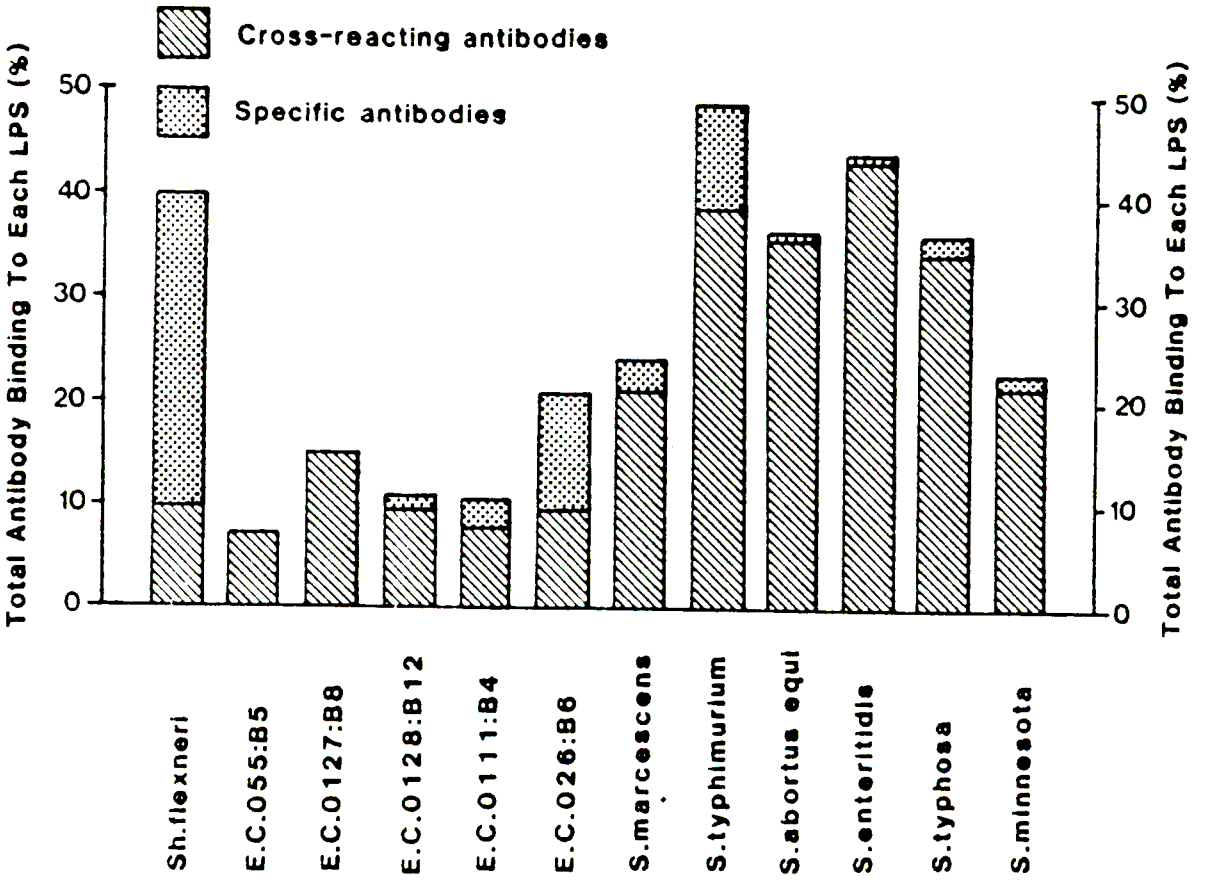
Cross-reacting antibodies
Specific antibodies
Total Antibody Binding To Each LPS (%)
50
40
30
20
10
0
Sh.flexneri
E.C.055:B5
E.C.0127:B8
E.C.0128:B12
E.C.0111:B4
E.C.026:B6
S.marcescens
S.typhimurium
S.abortus equi
S.enteritidis
S.typhosa
S.minnesota

Fig. 2

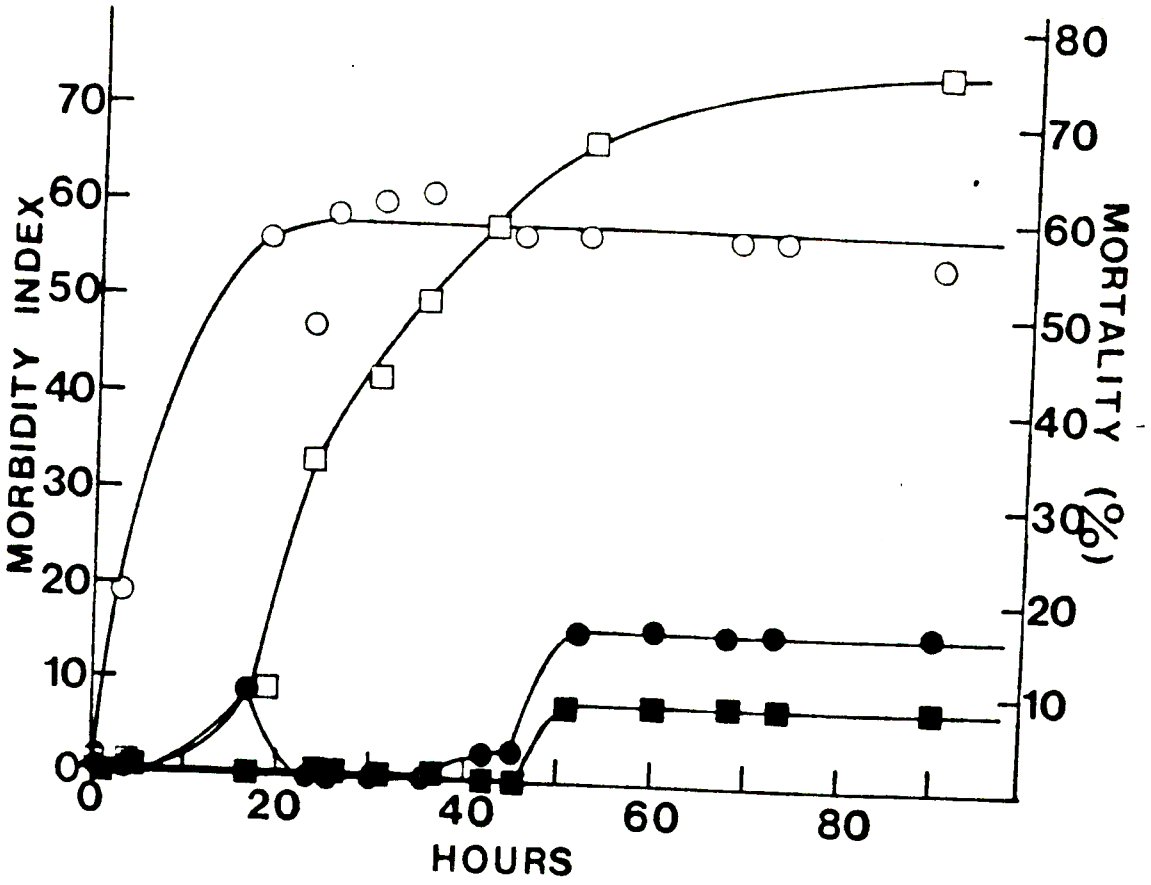
MORBIDITY INDEX
MORTALITY (%)
HOURS
0
20
40
60
80
10
30
50
70

FIG. 3

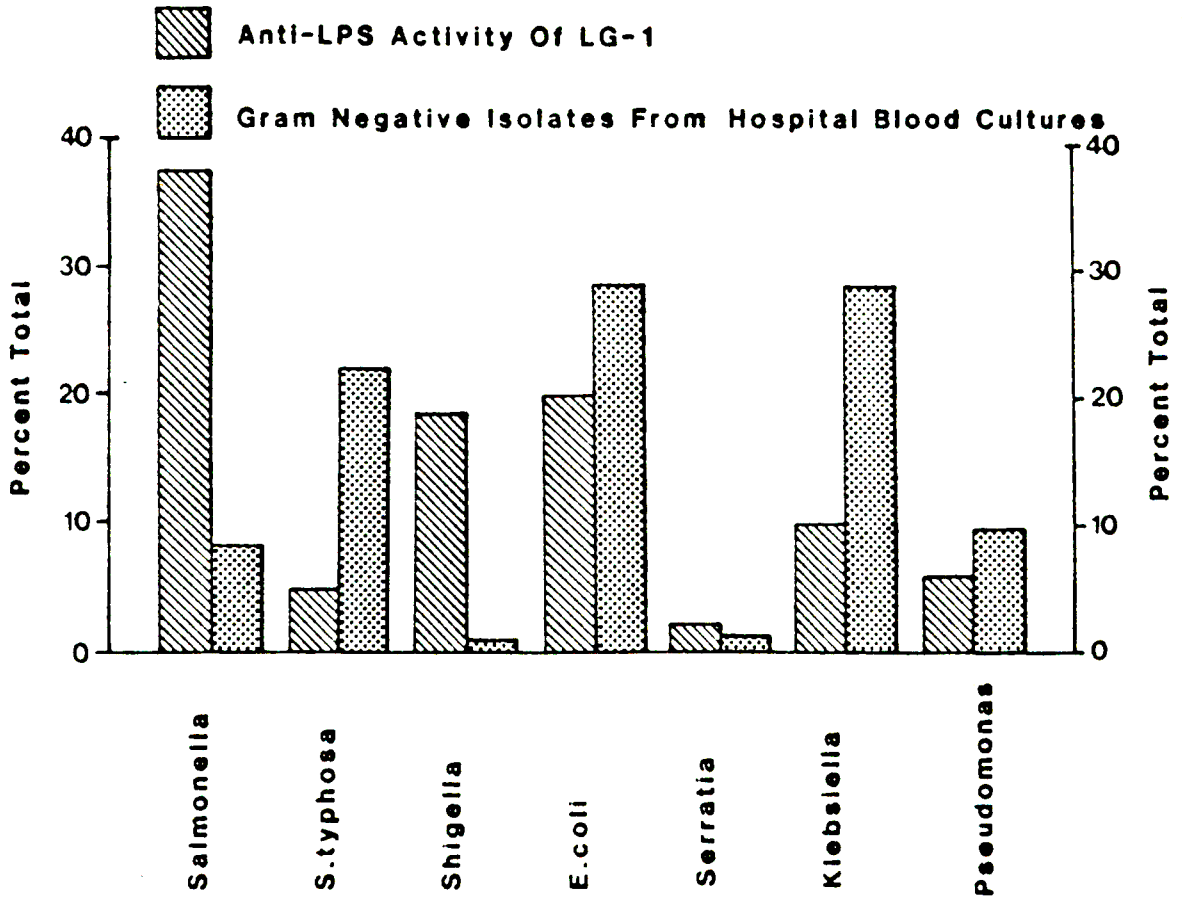
Anti-LPS Activity Of LG-1
Gram Negative Isolates From Hospital Blood Cultures
Percent Total
Percent Total
Salmonella
S.typhosa
Shigella
E.coli
Serratia
Klebsiella
Pseudomonas

FIG. 4.