CROSS-REACTIVE ANTIBODIES TO LIPOPOLYSACCHARIDE

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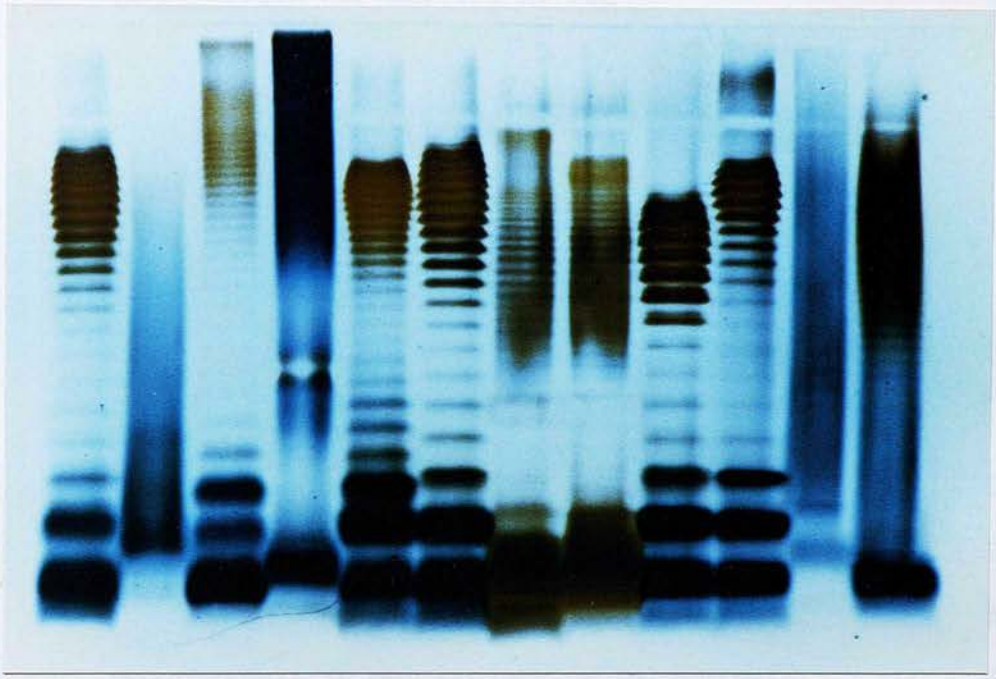


Frontispiece

Silver stained polyacrylamide gel of LPS (proteinase K digests) from 12 blood-culture isolates.

Lane a = 8b226 (*Escherichia coli*); b = 8b239 (*Proteus sp*); c = 8b257 (*Enterobacter cloacae*); d = 8b273 (*Serratia sp.*); e = 8b275 (*Escherichia coli*); f = 8b281 (*E. coli*); g = 8b300 (*Pseudomonas aeruginosa*); h = 8b305 (*Pseudomonas aeruginosa*); i = 8b316 (*E. coli*); j = 8b317 (*E. coli*); k = 9b24 (*Proteus sp.*); l = 9b 26 (*Klebsiella pneumoniae*).

CONTENTS

ABSTRACT

Lipopolysaccharide (LPS), also known as endotoxin, is a constituent of the outer membrane of Gram-negative bacteria which is toxic for humans and other animals. LPS probably plays a key part in the pathogenesis of Gram-negative bacteraemia and sepsis syndrome in humans. Cross-reactive antibodies to LPS may play a part in natural host defences, and may also be useful in the treatment of Gram-negative bacteraemia and sepsis syndrome. The structure of LPS, its toxicity, its role in Gram-negative bacteraemia and sepsis syndrome in humans, and the potential value of cross-reactive antibodies to LPS are reviewed.

The antibody response in recipients of typhoid vaccine was studied, with particular reference to the possibility that typhoid vaccine might induce the production of antibodies to the core region of LPS (LPS-core). In most recipients however the response observed was directed against specific antigens.

Urine samples from patients with suspected UTI were tested for IgG antibodies to LPS-core. Such antibodies were found to be associated with the presence of bacteriuria, although the association was not strong enough for antibody assay to be useful as a diagnostic test. Total urinary IgG was equally strongly associated with bacteriuria. This suggested that the antibodies were probably present because of non-specific leakage of serum components into the urine as a result of inflammation.

A large number of murine monoclonal antibodies (MAbs) to LPS-core had been produced by a collaborative group in Edinburgh and Basel, in the hope of producing a cross-reactive MAb which would be useful

therapeutically. The binding of some of these MAbs to a collection of blood-culture isolates of Gram-negative bacteria was studied. The principal method used was immunoblotting with LPS prepared by proteinase K digestion. These studies showed that, in general, the MAbs bound to *E. coli* but not to other Enterobacteriaceae commonly isolated from blood-cultures.

There was a suggestion from initial studies with MAbs that some were specific for particular core types of *E. coli*. This was investigated by reacting 79 blood-culture isolates of *E. coli*, together with control strains, with 61 MAbs in an enzyme-linked immunosorbent assay (ELISA). This allowed the identification of MAbs specific for *E. coli* core types R1, R2, and R3. The specificity of these MAbs for LPS-core was confirmed by immunoblotting. The MAbs were used to determine the core-type of *E. coli* strains from blood, urine, and faeces. The association of core type with O type, K type, serum resistance, and susceptibility to rough-specific phage was studied. R1 was the commonest core type among *E. coli* from all sites, and was associated with the common O types.

Some cross-reactive MAbs had been produced which, unexpectedly, did not react with LPS-core. Three of these MAbs were investigated. One appeared to react with enterobacterial common antigen. The other two reacted with a low-molecular weight protein, probably the Braun lipoprotein.

Small quantities of HA-1A (Centoxin), a human MAb which is said to react with lipid A, became available towards the end of the project. The MAb bound to a variety of materials, including some Gram-positive bacteria, and could not be shown to react with LPS.

DECLARATION

This thesis was composed entirely by myself, but laboratory studies were carried out within a research group.

In chapter 4 (typhoid vaccinees) I organised the collection of sera, and carried out the Widal tests. The ELISAs were performed by Linda Milne in the Microbial Antibodies Laboratory of the Blood Transfusion Service, Edinburgh. In chapter 5 (urine antibodies) I planned the work, collected the specimens, and performed pilot assays. The assays reported were carried out by two final-year honours students (Diane Edmond and Paul Stewart) working under my direction. I performed all the statistical analysis and produced all the figures. In chapter 6 (initial studies with MAbs) I carried out all the work described, except that Bill Neil of the Department of Medical Microbiology operated the flow cytometer. In chapter 7 (core-typing) the three ELISA assays of large panels of bacteria against large panels of MAb were carried out by Linda Milne and others in the Microbial Antibodies Laboratory of the Blood Transfusion Service, Edinburgh, and the correlation-sorting of these results was carried out by DR GR Barclay of that laboratory. The bacterial preparations for these assays were produced by Frances McLoughlin of the Department of Medical Microbiology, following a protocol I designed. All other assays including confirmatory ELISA work, immunoblotting, O-typing by ELISA, phage sensitivity and serum sensitivity were carried out by myself. In chapter 8, all the work described was carried out by myself except that both the Triton preparation and the phenol-water preparation of *E. coli* O:18 LPS were prepared by two final-year honours students under the supervision of Dr IR Poxton.

ACKNOWLEDGEMENTS

Thanks are due to many people have helped me with this thesis.

Ian Poxton was always optimistic and gave much encouragement and advice. Robert Brown was tolerant, and a mine of information on laboratory technique. Robin Barclay was endlessly enthusiastic and always helpful.

Frances McLoughlin, Linda Milne, Jan McColm, Bill Neil, Diane Edmond and Paul Stewart all assisted with the laboratory work, as detailed in the Declaration, and all were a pleasure to work with.

The staff of the Medical Illustration department, particularly Dave Ian and Lisa, performed miracles with invisible blots and broken gels, and produced all the photographic prints, always with a smile.

In the background are an army of support staff in media preparation, clinical bacteriology, antibody production, the decontamination area, and many others without whom this work would have been impossible.

I am also grateful to Professor JG Collee, Dr JF Peutherer, and Dr RS Miles and colleagues in clinical bacteriology for allowing me the time to pursue this research.

Funding for consumables came from Sandoz Pharma, and my salary from Lothian Health Board.

Chapter 1

INTRODUCTION

Lipopolysaccharide (LPS) is an essential component of the outer membrane of Gram-negative bacteria. It is a molecule which has attracted an enormous amount of scientific attention because of its toxicity to humans and other mammals, and in this context it is referred to as "endotoxin". LPS (or endotoxin) may be an extremely important mediator in Gram-negative bacteraemia and sepsis syndrome, and for this reason may be a key target for therapies aimed at such diseases.

This thesis is concerned with cross-reactive antibody to LPS. The experimental work consists of studies of the binding of antibodies to LPS. To put this work in perspective the introduction reviews the structure of LPS, its toxicity, the evidence that this toxicity may be important in Gram-negative bacteraemia and sepsis syndrome, and the evidence that cross-reactive antibodies to LPS may be beneficial.

LPS consists of three regions: lipid A, core oligosaccharide, and O polysaccharide, as shown schematically in figure 1.1. Lipid A, which is the innermost part of the molecule, is the most conserved part, and the part most clearly associated with toxicity. The O polysaccharide, which forms the outer part of the molecule, is the most variable and the most immunogenic. The core region, which is central to the molecule also lies metaphorically between lipid A and O polysaccharide with regard to immunogenicity, variability and association with toxicity. Three major reviews of LPS structure have been published recently [29,142,193].

LIPID A

Lipid A forms the inner-most part of the molecule and is inserted into the outer leaflet of the outer membrane of Gram-negative bacteria. Lipid A makes up a large proportion of the outer leaflet, and must be of structural and functional significance to Gram-negative bacteria.

The chemical structure of lipid A from *E. coli* is shown in figure 1.2. It has a hydrophilic backbone made up of a 1,4'-bisphosphorylated β-1,6-D-glucosaminyl-D-glucosamine disaccharide. Four acyl residues are attached directly to the glucosamine residues, and two of these acyl residues are themselves esterified with further acyl groups, forming acyloxyacyl residues.

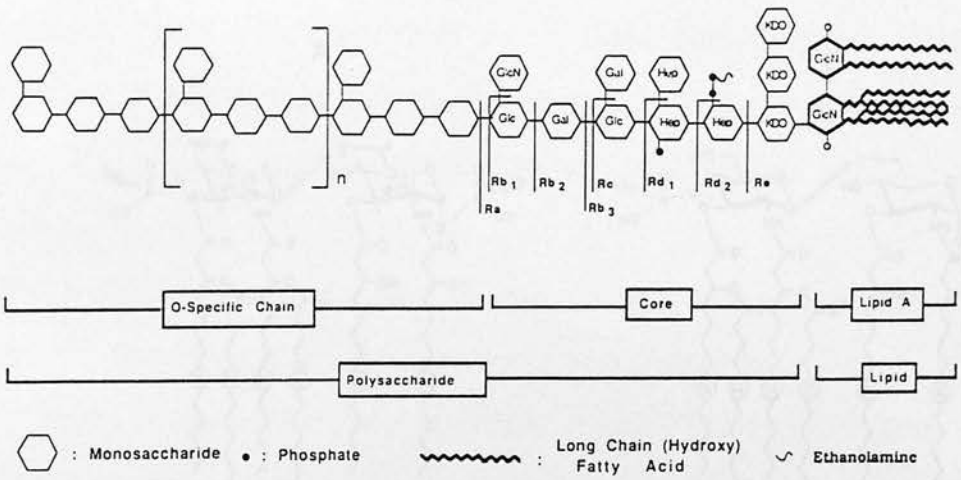


Figure 1.1 Schematic diagram of lipopolysaccharide structure [130].

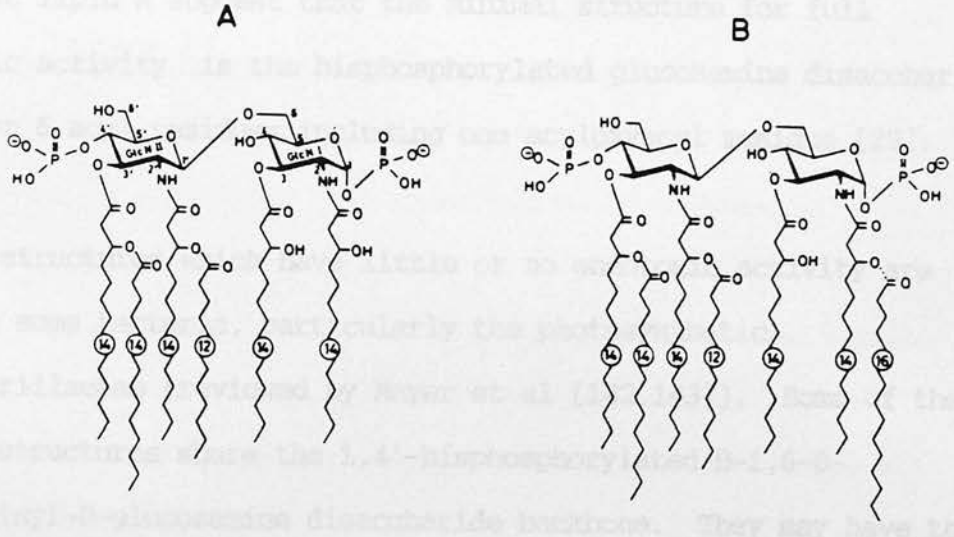


Figure 1.2 Covalent structure of lipid A from *E. coli* (A) and *Salmonella minnesota* (B) [195]. Numbers in circles refer to the number of carbon atoms in the acyl chains.

Lipid A from other organisms shares the same overall structure. Among the endotoxic lipid A structures from enterobacteriaceae, structural variations concern the number of carbon atoms in the hydrocarbon chains, the type and distribution of the acyloxyacyl residues, and additional substitution of the phosphoryl residues. *Salmonella* lipid A, for example, has an additional 16 carbon acyl group forming a third acyloxyacyl group, and the two phosphoryl groups are substituted with phosphorylethanolamine and 4-deoxy-4-amino-L-arabinopyranose respectively (figure 1.2). Studies with synthetic lipid A suggest that the minimal structure for full endotoxic activity is the bisphosphorylated glucosamine disaccharide with 5 or 6 acyl residues including one acyloxyacyl residue [29].

Lipid A structures which have little or no endotoxic activity are found in some bacteria, particularly the photosynthetic Rhodospirillaceae (reviewed by Mayer et al [142,143]). Some of these lipid A structures share the 1,4'-bisphosphorylated β-1,6-D-glucosaminyl-D-glucosamine disaccharide backbone. They may have the amide-linked 3-hydroxy fatty acids replaced partially or completely by 3-oxo fatty acids, as is found in *Rhodobacter* spp.. Alternatively the glucosamine disaccharide may have an additional glucosamine residue attached, as in *Rhodocyclus tenius*. *Bacteroides fragilis* Lipid A, which is 100 to 1000 times less toxic than lipid A from *Enterobacteriaceae*, lacks one of the phosphate residues and has an average of only 5 fatty acid residues per glucosamine disaccharide [131].

A further group of lipid A variants with a backbone containing one or two residues of diaminoglucose are referred to as lipid A_{DAG}. Lipid A_{DAG} has been found in at least 25 different species, including some pathogens such as *Brucella abortus* [160]. The disaccharide form of lipid A_{DAG}, as found in *Pseudomonas diminuta* [107] has endotoxic properties, while the monosaccharide form, as found in *Rhodopseudomonas viridis* [77], does not.

LPS-CORE

Attached to the C6 hydroxy residue of the non-reducing glucosamine of lipid A is the core oligosaccharide or "LPS-core". The structure of LPS-core can be subdivided into inner and outer regions.

The inner core typically consists of two or three KDO (keto-deoxy-octulosonic acid, now known as 3-deoxy-D-manno-2-octulosonate) residues and two or three heptose residues. The KDO residues are linked together, one of them being directly linked to lipid A. The inner core is, like lipid A, highly conserved, at least among Enterobacteriaceae, and *Pseudomonas*. There is however variation in the inner core structures of widely divergent bacteria, as shown in figure 1.3. In some species, such as *Acinetobacter calcoaceticus* [111], a KDO analogue is thought to occupy the linking position between lipid A and the outer core, but KDO is present also.

The outer core is typically made up of 3, 4 or 5 hexose and hexosamine residues, and is more variable than the inner core. There are 4 variants (termed R1 - R4) described in wild-type *E. coli*, and

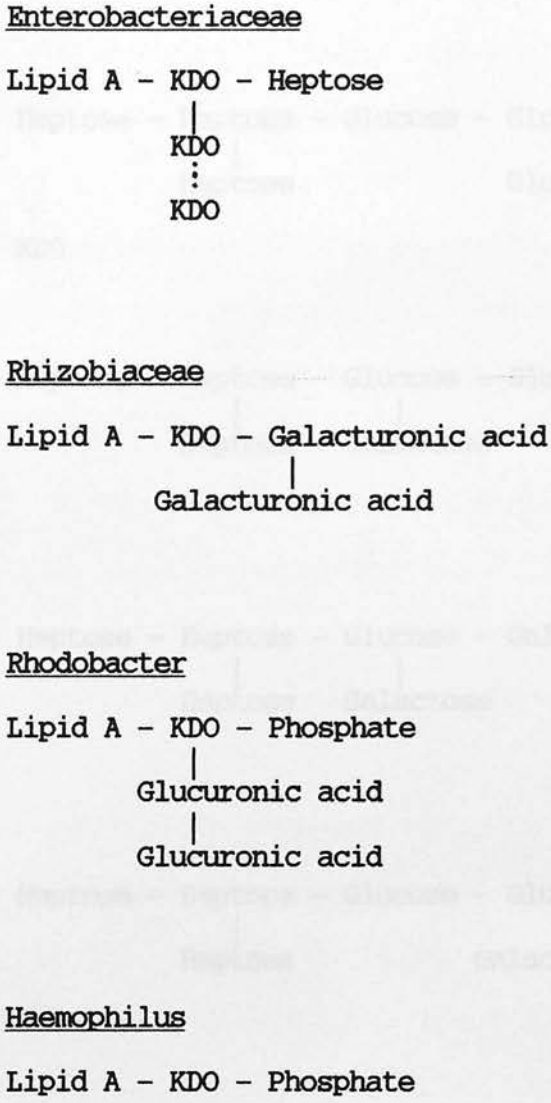
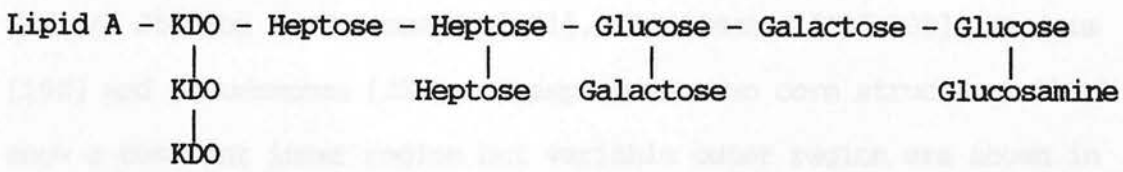
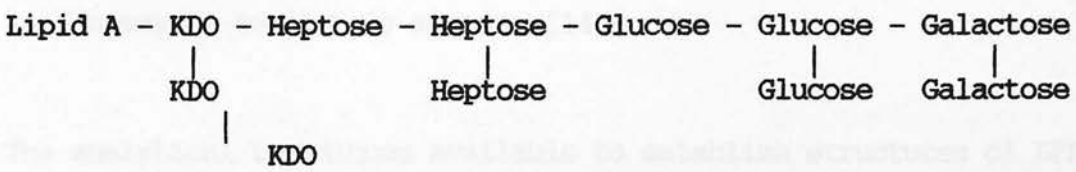


Figure 1.3 Covalent structures of the inner core region [142].

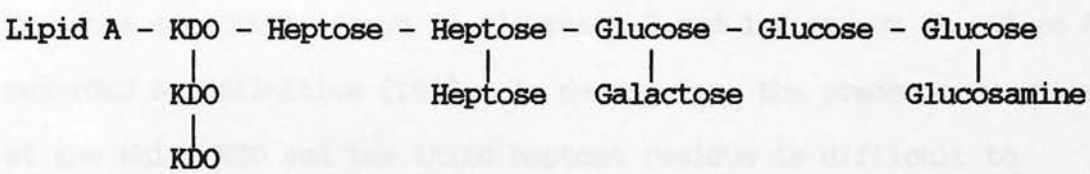
Salmonella



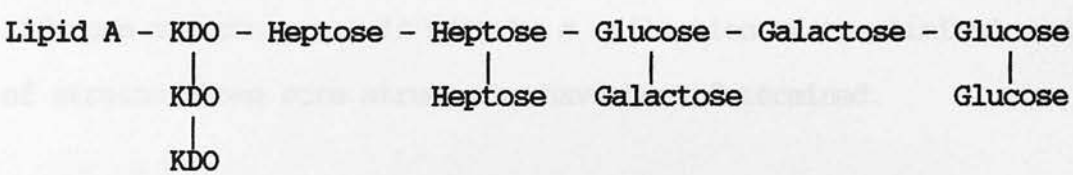
E. coli R1



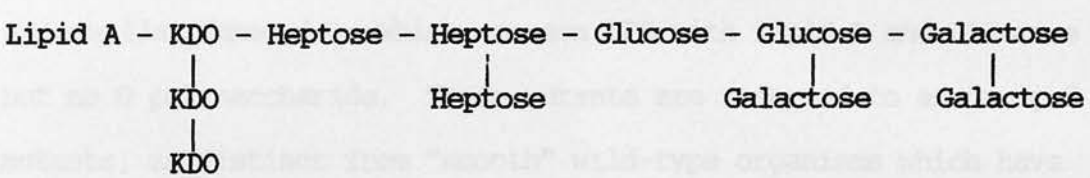
E. coli R2



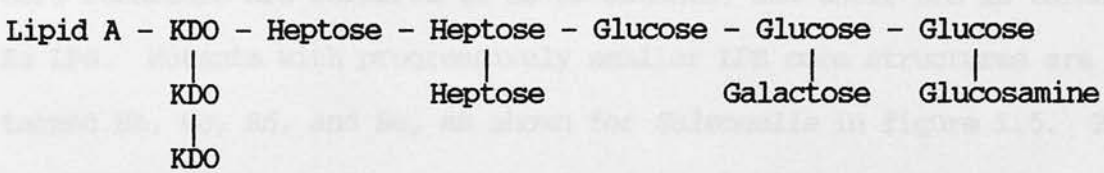
E. coli R3



E. coli R4



E. coli K12



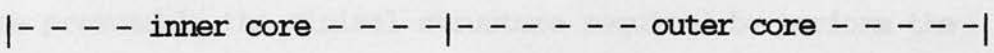


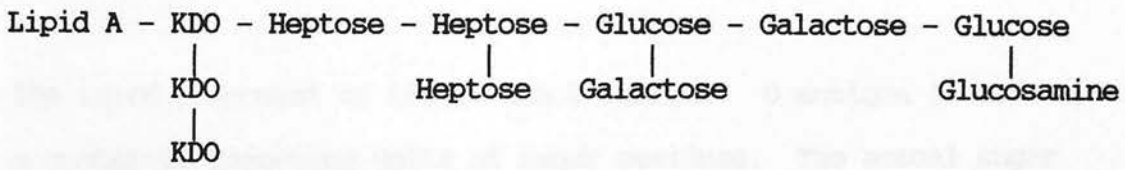
Figure 1.4 Covalent structures of LPS-cores of *E. coli* and *Salmonella* [101].

other variants noted in "laboratory" strains of *E. coli* (eg *E. coli* K12 and J5) and in *Salmonella* [101], *Citrobacter* [197,198], *Proteus* [192] and *Pseudomonas* [200]. Examples of some core structures which show a constant inner region but variable outer region are shown in figure 1.4. In some non-enterobacterial species the outer core region may be completely missing [142].

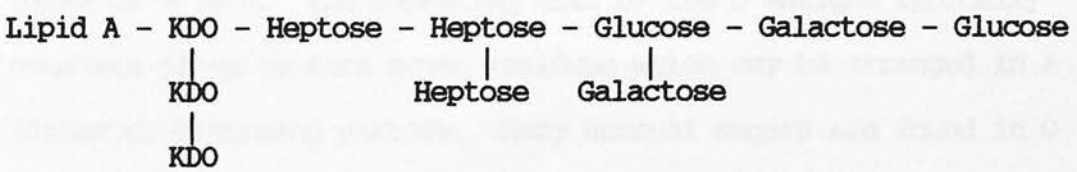
The analytical techniques available to establish structures of LPS-core in intact LPS are difficult, indirect, and subject to artifacts. The core structures shown in figures 1.3 and 1.4 cannot therefore be regarded as definitive [193]. In particular, the presence or absence of the third KDO and the third heptose residue is difficult to establish. It should also be noted that the apparent conservation of LPS-core structure may in part be a reflection of the limited number of strains whose core structures have been determined.

Mutants have been obtained from several bacteria (notably *E. coli* and *Salmonella minnesota*) which express LPS with lipid A and LPS-core but no O polysaccharide. These mutants are referred to as "rough" mutants, as distinct from "smooth" wild-type organisms which have complete LPS structures. Rough mutants which have a complete LPS-core structure are referred to as Ra mutants, and their LPS is termed Ra LPS. Mutants with progressively smaller LPS core structures are termed Rb, Rc, Rd, and Re, as shown for *Salmonella* in figure 1.5. Re LPS is the smallest LPS structure found in viable bacteria, and therefore presumably the minimal structure required to perform the essential functions of LPS. Re LPS always contain lipid A and either one (or more usually two) KDO residues, or occasionally, (as in *Acinetobacter*, [30]) a KDO-analogue.

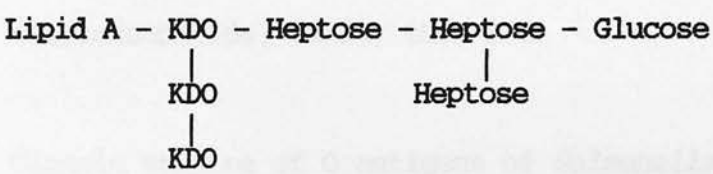
Ra



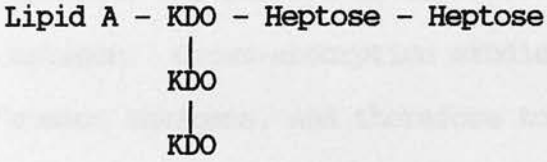
Rb



Rc



Rd



Re

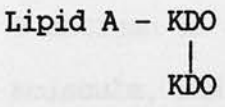


Figure 1.5 Covalent structures of the LPS-core region of rough mutants of *Salmonella* [29].

O ANTIGEN

The third component of LPS is the O antigen. O antigen is made up of a number of repeating units of sugar residues. The actual sugar residues involved varies considerably among strains of even a single species of bacterium. For example there are some 150 different O types of *E coli*. The repeating unit of the O antigen typically consists of up to five sugar residues which may be arranged in a linear or branching pattern. Many unusual sugars are found in O antigens which are rarely found elsewhere [142]. In some organisms the O polysaccharide is absent or consists of only one or two repeating units, in which case the molecule is called LOS (Lipo-oligosaccharide) rather than LPS.

Classic studies of O antigens of *Salmonella* and *E. coli* [110] involved analysis of the carbohydrate constituents of LPS and antisera produced in rabbits. The cross-reactions between sera were studied, and related to the carbohydrate constituents of the O antigen. Cross-absorption studies were used to remove antibodies to common antigens, and therefore to identify specific O "factors" within the O antigen. These factors form the basis of the Kauffman-White scheme for the classification of *Salmonella*.

O antigen is thought of as the immunodominant part of the LPS molecule, and indeed may be the dominant antigen of many Gram-negative bacteria. The term "immunodominant" sugar was first used [134] to refer to the best inhibitor of a factor-antifactor reaction. Cross-absorption experiments of the type used to prepare antifactor

sera would effectively remove antibody to less diverse antigens such as LPS-core. The concept was therefore not based on an objective comparison of the responses to O antigen and other components of the organism, such as LPS-core.

MOLECULAR MODELLING OF LPS

The likely three-dimensional structures of Re LPS from *E. coli* have been calculated by Kastowsky et al [109]. In all, 19 different models consistent with the insertion of Re LPS into a highly ordered membrane were obtained. In all cases the diglucosamine backbone was found to be tilted by 53 ± 7° from the membrane normal, while the two glucosamine residues were parallel to the membrane normal. The calculated conformation of the two KDO residues was sensitive to the presence of cations, and could be either rigid (in the presence of divalent cations) or mobile (in the presence of monovalent cations). Similar calculation for a whole LPS molecule [108] suggest that the conformation of LPS-core is stable and roughly cylimdrical. Some groups (e.g. some of the KDO carboxyl groups) which may be exposed in Re LPS are buried within the molecule. The O-specific chain, in contrast, has a very variable conformation.

Three-dimensional structures have also been calculated for the various outer core structures of *E. coli* and *Salmonella*, based on the accepted primary structures [103]. These calculations tend to suggest that the different core structures assume a similar overall conformation, despite the variation in sugar content and linkages. This may point to the functional determinants of LPS-core structure.

Consideration of three-dimensional structures is helpful in considering the epitopes to which antibodies to LPS-core may bind, as discussed in chapter 6. Molecular modelling may help to clarify the function of LPS both as a structural element in the bacterium and as endotoxin in the mammalian host.

INTRA-STRAIN VARIABILITY OF LPS

As well as variation between species and strains, there may be variation in the structure of LPS obtained from a single bacterial isolate. LPS prepared from a single bacterial culture shows heterogeneity in the length of the O polysaccharide chain, and includes some molecules completely lacking in O polysaccharide . Electrophoresis of LPS preparations from a single smooth bacterium in a polyacrylamide gel (frontispiece) yields a typical "ladder" pattern representing this heterogeneity [180]. The fastest moving band (shown at the bottom of the gel) consists of lipid A and LPS-core with no O antigen, while each successive band above that represents LPS with the addition of one more O antigen repeating unit.

Within lipid A and core there may also be variation, or "microheterogeneity" in the structures, which causes additional problems in attempts to define structures [142]. This may consist of incomplete substitution of some of the branching groups such as phosphate.

In *Pseudomonas aeruginosa* there are two distinct forms of LPS present in wild-type strains. These two forms differ in the nature of the O

repeating unit. The O polysaccharide of form A is the same in most *Pseudomonas aeruginosa*, while the O polysaccharide of form B is variable and accounts for the O typing antigen [125].

Conditions of growth may also affect the nature of the LPS produced by an individual organism. Nutrient limitation may increase the proportion of LPS molecules which lack O polysaccharide [165]. Duration of culture (or growth phase) can also alter the antigenicity and toxicity of isolated LPS [153]. In *Yersinia enterocolitica* the nature of the LPS expressed depends on the growth temperature and calcium concentration [50]. Growth conditions may therefore be crucial to the laboratory production of LPS for use in experiments which attempt to model clinical infection.

LPS, also known in this context as endotoxin, is toxic to humans and other animals. This toxicity was initially observed following injection into animals, and the manifestations observed were fever, shock, death. More complex manifestations, such as the Shwartzman reaction and induction of tolerance may also be observed in whole animals. Unlike classic protein exotoxins there is no single specific action, but many different mechanisms which have been demonstrated in a wide variety of in-vitro systems [201]. These actions include the activation of complement and coagulation factors, and the stimulation of platelets (in rabbits), macrophages, neutrophils [61], and endothelial cells [250]. Assessing the significance of these actions of endotoxin is complicated by the many secondary effects that are produced, for example by activated complement components.

Lipid A appears to be the minimal LPS structure required for endotoxic effect [29,75,193]. Free lipid A is however insoluble and lacks biological activity, but can be made soluble and biologically active by removing the cations which neutralise the negatively charged groups. O antigen and LPS-core are not in themselves toxic, but they do render the molecule water soluble (at least as micelles) and thus modulate the biological activity of lipid A, for example increasing the circulating life of LPS [73].

Early research on endotoxin stressed the importance on mediators released from leukocytes [256]. Modern researchers have returned to

this view [51,82,83], despite the demonstration of other actions of endotoxin. The key cell is thought to be the macrophage, which is triggered by endotoxin to produce cytokines which in turn are responsible for the toxic effects.

The importance of macrophages is strongly supported by studies of a strain of C3H/HeJ mice (LPS^d) which are genetically resistant to LPS. Transfer of macrophages from the LPS sensitive LPS^n mice restore LPS sensitivity in the galactosamine-sensitised model of endotoxicity [74].

Evidence for the central role of cytokines has been reviewed in detail recently [83,136,240]. Experimental endotoxaemia in animals and man leads to the appearance of Tumour Necrosis Factor (TNF), Interleukin (IL) -1 and IL-6 in the circulation, and these cytokines may also be detected in the circulation of patients with sepsis. The major source of these cytokines is thought to be the macrophage. TNF, IL-1, and IL-6 have been produced by recombinant DNA technology and have all been shown to be pyrogenic in animals. High doses of TNF or IL-1 independently can produce shock, and act synergistically when given together, leading to all the pathological features typical of endotoxic shock. TNF has also been shown to produce endotoxin-like effects in human volunteers. Antibodies to either IL-1 or TNF can protect against the toxic effects of endotoxin, as can a variety of other agents which block the production or action of these cytokines. TNF, IL-1 and IL-6 appear to interact with one another in a variety of ways. TNF and IL-1 stimulate the production of each other and of IL-6, while IL-6 impairs endotoxin-induced TNF and IL-1 production.

These interactions may explain why blockage of either TNF or IL-1 action is sufficient to block endotoxic effects.

The stimulation of macrophages to release cytokines probably involves specific receptors (Reviewed by Wright [260]). Three classes of receptor on macrophages can recognise endotoxins. Two of these (CD18 and the scavenger receptor) mediate uptake and catabolism of the LPS. The third receptor, CD14, recognises complexes of LPS with the serum protein lipopolysaccharide binding protein (LBP). Binding of the LPS/LBP complex to CD14 appears to trigger the synthesis and secretion of TNF. The existence of specific receptors is compatible with finding that some lipid A analogues [247,248] and natural non-toxic lipid A structures [84] can block the effects of endotoxin.

The stimulation of macrophages to produce Interleukins and TNF cannot explain all the effects of endotoxin. For example, the finding that a monoclonal antibody can block the lethal effect of experimental challenge with *E. coli* in mice without altering the pattern of TNF and IL-6 levels [218] points to the existence of other significant pathways. Among the many possible targets most current interest relates to the release of nitric oxide from endothelial cells [250] and interactions with neutrophlis and endothelial cells in the lung [61].

Although conventionally thought of as a toxin, it is also possible to regard LPS as the primary signal which animals use to trigger a protective response to invasive Gram-negative infection [127]. From this viewpoint the host response to endotoxin is seen as a well-

adapted protective system with positive and negative feed-back loops which ensure a rapid but controlled and normally beneficial response. Harmful effects are seen as an overactive response occurring when bacterial invasion is not initially controlled, for example in experimental sepsis where large numbers of organisms are injected into an immunocompromised host. This viewpoint explains why evolution has favoured the retention of the response to endotoxin when single mutations [74] can lead to a greatly reduced response.

Much has been learned about the mechanisms by which endotoxin may produce its effects. Much more remains to be learned about when particular mechanisms are triggered and about the possible benefits of medical interventions aimed at blocking these mechanisms.

ENDOTOXIN IN GRAM-NEGATIVE BACTERAEMIA AND THE SEPSIS SYNDROME.

Endotoxin is thought to be the key microbial element in the causation of the systemic features of Gram-negative bacteraemia. Many other properties of the organisms involved may be important in colonising and invading the host, and in aiding microbial survival in the face of attack by the host defences. A few Gram-negative pathogens also produce protein exotoxins which are important in pathogenesis. It is endotoxin however which is thought to be the cause of the symptoms observed in most cases [261]. Gram-negative bacteraemia will be reviewed in some detail because of the doubt that has arisen over its significance [116,137], and therefore over the significance of endotoxin derived from bacteraemic organisms.

Endotoxin absorbed from the gut may also be an important cause of illness [242]. This may occur in the presence or absence of bacteraemia.

GRAM-NEGATIVE BACTERAEMIA

The role of Gram-negative bacteraemia in systemic disease has been studied principally in two overlapping groups of patients: patients with bacteraemia, and patients with clinically defined illness. Studies of series of patients with Gram-negative bacteraemia has shed useful light on which particular organisms are involved and (to a limited extent) why. The weakness in these studies is an underlying assumption that the organisms are clinically important, an assumption which is often not tested. Studies of patients who have sepsis

syndrome or septic shock (systemic illnesses of the type which could be produced by endotoxin) allow a broader view of the possible causes, and raise the question of whether the Gram-negative bacteria in the blood are important.

Failure to appreciate the differences between the two groups of patients has led to some misinterpretation of the data. For example Glauser et al [83] refer to the series of patients reported by Ispahani et al [99] as a series of septic shock patients when in fact they are a series of cases of bacteraemia. This leads to the false impression that bacteraemia is present in most cases of septic shock.

Gram-negative bacteraemia is a condition which is now common in hospitalised patients in Western countries. The frequency has increased and the spectrum of organisms has changed during this century. The organisms involved include some specific pathogens (eg *Salmonella typhi*, *Haemophilus influenzae*, and *Neisseria meningitidis*), but are now mostly gut commensals (particularly *E. coli*), acting as opportunist pathogens.

In 1924 *Bacillus coli* (≡ *E. coli*) was "one of the least frequently encountered" organisms in blood cultures in septic disease [67]. Common organisms at that time were Gram-positive cocci and *Salmonella typhi*. The changes since then have not been widely recorded, but one major hospital (Boston City Hospital; [155]) has kept consistent and detailed records of Gram-negative bacteraemia over the period 1935 to 1972 and observed an increasing trend. More recently attempts have been made in the USA to estimate the number of cases of septicaemia

(defined as "systemic disease associated with the presence and persistence of pathogenic microorganisms or their toxins in the blood") diagnosed each year in hospitals in the USA. The numbers of cases more than doubled in the period 1979-1987 [2], and although the organisms responsible were not reported in this study it seems likely that the number of Gram-negative bacteraemias must be continuing to rise.

From 1935 to 1972 the proportion of Gram-negative bacteraemias which were due to *E. coli* fell, despite an increase in absolute numbers. The proportion of other organisms, notably *Klebsiella* spp., increased [155]. A review of large series of Gram-negative bacteraemia reported between 1965 and 1987 [261] showed that *E. coli* was still the commonest single isolate, with *Klebsiella* spp. second and *Pseudomonas aeruginosa* third, and this pattern is maintained in more recent series [66,72,99,168]. A number of other genera are consistently reported, notably *Enterobacter, Acinetobacter, Proteus* and *Citrobacter*.

Table 1.1 shows figures from one series [168], which illustrate some further points about the organisms involved in Gram-negative bacteraemia. First, there is a difference between community-acquired infections and nosocomial (hospital acquired) infection, with a greater proportion of *E. coli* in the community-acquired cases and a greater proportion of *Klebsiella* spp., *Enterobacter* spp., *P. aeruginosa* and *Acinetobacter* spp. in the hospital-acquired cases. Second, this series contains a relatively large number of primary pathogens among the community-acquired cases. In the case of

Table 1.1 Gram-negative bacteria isolated from blood cultures in Columbia-Presbyterian Medical Centre, New York, in a 20-month period in 1988-89 [168].

Organism	Number		
	Community-acquired	Nosocomial	Total
E. coli	148	56	204
Klebsiella spp.	62	65	127
Pseudomonas aeruginosa	14	52	66
Enterobacter spp.	14	33	47
Acinetobacter spp.	6	40	46
Haemophilus influenzae	23	0	23
Bacteroides spp.	13	9	22
Proteus spp.	10	8	18
Pseudomonas spp.	8	7	15
Serratia spp.	4	4	8
Citrobacter spp.	1	5	6
Salmonella typhi	5	0	5
Haemophilus spp.	0	4	4
Morganella spp.	2	2	4
Achromobacter spp.	3	1	4
Neisseria spp.	3	0	3
Other	4	1	5
Total	320	287	607

Salmonella typhi this must reflect a high prevalence of this organism in the population served. In the case of *Haemophilus influenzae* (presumably Pitmann type b) it probably reflects a large paediatric service in the hospital. This study [168] also found an increase in the proportion of *Acinetobacter* spp. between 1981 and 1988, indicating that change in the spectrum of organisms is continuing. It is likely therefore that there will continue to be differences between hospitals and over time in the range of organisms causing Gram-negative bacteraemia.

E. coli, the commonest single cause of Gram-negative bacteraemia, has been studied in detail. A small proportion of the many O:K:H types described account for most cases of *E. coli* bacteraemia [39,52,177]. The O types involved are broadly the same as those commonly found in urinary infections and as faecal commensals [89,246], though there appear to be geographical and temporal variations. The O types found in bacteraemia are different from those recognised as enteropathogenic strains [178]. Most *E. coli* isolated from blood cultures are resistant to killing by fresh serum [52], a property which is clearly likely to favour the survival of extracellular bacteria in the blood. Serum resistance is itself associated with some O types and with the presence of K1 capsule, though K1 capsule is found no more commonly in bacteraemic strains than in faeces [196].

The organisms causing Gram-negative bacteraemia probably originate most commonly from the gut. These organisms can establish foci of infection in various sites, depending on the predisposition of the

patient. The most commonly identified foci of infection in the series cited above are in the urinary tract, the abdomen, or (following overgrowth of coliforms in the pharynx [104,138]) in the respiratory tract. The differences observed between organisms involved in community acquired and hospital-acquired infection are thought to be the result of a replacement of endogenous gut flora by bacteria in the hospital environment [186,215,243], as a result of antibiotic and other pressures. The predominance of *E. coli* in community-acquired Gram-negative bacteraemia therefore reflects its presence as the predominant aerobe in the normal gut flora, while the higher proportion of *Klebsiella* and *Pseudomonas* spp. in hospital acquired infections reflects the replacement of gut flora by these organisms. *Acinetobacter* spp. may be an exception to this generalisation, however, since the commonest sites of infection identified for this organism are intravenous lines [168]. The increase in *Acinetobacter* spp. as a cause of bacteraemia may therefore simply be a result of increased use of intravenous lines.

Mortality in patients with Gram-negative bacteraemia has been variously reported at between 20 and 50% [261]. With the exception of cases caused by invasive pathogens such as *Salmonella typhi*, Gram-negative bacteraemia is a complication of an underlying disease process rather than a primary cause of disease, and the observed mortality is therefore in part a reflection of the underlying disease. In the meta-analysis of Young (1990), mortality rates were highest for *P. aeruginosa*, and second for *Klebsiella* spp., with *E. coli* coming in fourth after the relatively infrequent *Proteus* spp.. Only *P. aeruginosa* bacteraemia has however been shown to be

independently associated with increased mortality [158], and even then the effect is weak. Most of the differences in mortality are explained by the association of *P. aeruginosa* and *Klebsiella* spp. with more severe underlying disease [118]. This is related to the differences observed between community-acquired and nosocomial infection, since the patients with nosocomial infection tend to be those with more severe and complicated underlying illness, and therefore a higher mortality rate [72,99].

Among *E. coli* bacteraemias, serum resistance and O types 4, 6, and 8 have been associated with higher mortality, but so have the absence of recognisable capsule and rough phenotype [151]. It is not clear whether these differences are due to increased virulence or association with more severe underlying illness.

It appears therefore that the various Gram-negative organisms which commonly cause bacteraemia are all approximately equal in the harm which they cause. This is compatible with the idea that the harm is caused by endotoxin, which is common to them all.

IS GRAM-NEGATIVE BACTERAEMIA A CAUSE OF ILLNESS?

It is possible to assume that Gram-negative bacteraemia is harmful. The patients involved have a high mortality, and there is ample evidence from studies of the action of endotoxin and from experimental infections in animals to conclude that the organisms could be responsible for the illness observed. There are two important inconsistent findings however. First, Gram-positive

bacteraemias, even those caused by coagulase-negative staphylococci, are associated with similar mortality rates to those associated with Gram-negative bacteraemias [72,99,139,252]. This calls into question the pre-eminent role of endotoxin as a bacterial virulence factor. Second, among patients with clinically defined sepsis syndrome, those with bacteraemia (Gram-negative or Gram-positive) fare no worse than those without bacteraemia [26,86,221,266]. The bacteraemia in these patients might therefore be just an epiphenomenon, with the real harm coming from some other stimulus to the cytokine release. The question of whether bacteria are important in sepsis syndrome has been the subject of a review [81], the key points of which are reproduced here.

Sepsis syndrome and Septic shock

The term "sepsis syndrome" has been used [23,24,83] to describe a condition of fever or hypothermia associated with tachypnoea, tachycardia, and evidence of inadequate tissue perfusion, in a patient with clinical evidence of infection. "Septic shock" is used to refer to a more severely ill group of patients who in addition to the features noted above, have signs of shock. These definitions could apply to a very wide range of patients, but are usually applied to patients in intensive care units in developed countries. Most such patients are suffering primarily from trauma, or from intra-abdominal infection which has not been eradicated surgically.

A consensus conference on the terminology to be used in this area [25] has recently suggested that a new and broader term, "Systemic Inflammatory Response Syndrome" is required to describe the clinical features of sepsis syndrome in patients who may or may not have underlying infection. The condition would be termed Sepsis "when the systemic inflammatory response syndrome is the result of a confirmed infectious process". An infection is defined as "an inflammatory response to the presence of microorganisms or the invasion of normally sterile host tissues by those microorganisms". This approach does seem to clarify the terminology, but it leaves unanswered the question of how to establish that an infectious process may cause the systemic inflammatory response.

Bacteraemia in sepsis syndrome

There have been four major prospective studies of patients with clinically defined sepsis syndrome [26,86,221,266] and one of septic shock [54]. Studies of subgroups of three of the sepsis syndrome series have been published separately, duplicating some of the data [27,232,259]. Two other studies [38,267] were prospective in design but appear to have included Gram-negative bacteraemia as one of the possible entry criteria, and cannot therefore be used to determine the frequency of bacteraemia in sepsis syndrome. In prospective series of sepsis syndrome patients, bacteraemia was found in 40-47% [26,221] and Gram-negative bacteraemia was found in 21-37% [26,86,221,266]. In the prospective study of septic shock, 39% had bacteraemia, of whom about half (19% of the total) had Gram-negative bacteraemia [54]. Full detail of the blood culturing protocol is not

given in any of these studies, and there may be false negatives in some series if inadequate volumes of blood were cultured before antibiotics were given [71]. Bacteraemia (Gram-positive or Gram-negative) was associated with the development of septic shock in one series of febrile patients [241] and in one series of sepsis syndrome patients [27], but not in the other series. Bacteraemia was not associated with a worse outcome in any of these series.

Evidence that bacteremia is harmful.

The ideal way to show that mortality or morbidity is attributable to Gram-negative bacteraemia would be to perform a case-control study. A study of this nature has demonstrated that bacteraemia due to coagulase-negative staphylococci results in increased mortality and prolonged hospital stay [139]. Such studies specifically dealing with Gram-negative bacteraemia do not exist, although mortality attributable to bacteraemia in general was demonstrated in neonates in a study where 70% of the bacteraemias were due to Gram-negative bacilli [234]. Other studies have attempted to distinguish, in a less formal way, mortality due to underlying disease from mortality due to infection [99,252].

Clinical studies of the effect of antibiotics do support the idea that bacteraemia is harmful. Since the demonstration that early antibiotic treatment led to greatly reduced mortality in *Pseudomonas* bacteraemia [205] antibiotics have been used empirically for patients in whom bacteraemia is suspected. It would be considered unethical now to withhold antibiotics as part of a double-blind study

to show their benefit, and therefore such definitive studies have not been done. More oblique approaches have been used however, including comparisons of different antibiotic regimens, and retrospective studies of the outcomes of individual patients.

Comparisons of antibiotic regimens.

Large studies in febrile neutropenic patients have compared different empirical antibiotic regimens [115,262], and have shown differences between outcome for the different regimens. These studies have also shown that *in-vitro* sensitivity tests on blood-culture isolates are good predictors of the effectiveness of treatment regimens. This implies that the differences in outcome observed are due to the antibiotic action of the drugs, and therefore that the living bacteria detected in the blood were important. However, in these patients, unlike sepsis syndrome patients, bacteraemia itself is a predictor of a worse outcome [115]. This difference is probably related to the much lower clinical threshold for admission of these patients to trials. These results therefore confirm that bacteraemia can be a cause of illness, but they are not necessarily applicable to sepsis syndrome patients in whom other more powerful insults may be responsible for the illness observed.

Retrospective series of bacteraemic patients.

Retrospective studies have looked at the difference in outcome for patients treated with appropriate antibiotics (antibiotics to which the organism isolated was sensitive) and those treated with

inappropriate antibiotics (to which the organism was not sensitive). Significantly lower mortality rates in patients receiving appropriate antibiotics were found in three large series of bacteraemic patients [119,185,252] and in several smaller series of patients bacteraemic with groups of organisms such as *Enterobacter* [19] *Klebsiella* [251] *E. coli* [85]. The relationship between appropriate antibiotic and outcome has been considered in one small study of sepsis syndrome patients [113], which confirmed that inappropriate antibiotics were associated with a significantly worse outcome. Delay in introduction of the appropriate antibiotic was also associated with a significant increase in hospital stay and with the development of acute organ failure, though not with increased mortality. This study considered appropriateness in relation to "the cultured pathogen", which presumably means isolates from abscesses and other sites as well as blood cultures, but nevertheless supports the view that antibiotics can be effective in sepsis syndrome patients, and therefore that invading organisms, such as blood culture isolates, are important.

Do antibiotics completely neutralise the deleterious effect of bacterial invasion?

The apparent effectiveness of antibiotic treatment offers an explanation for the failure to observe a worse outcome in sepsis syndrome patients who have bacteraemia. Among sepsis syndrome patients, 90.5% to 98% [86,266,267] of those with Gram-negative bacteraemia, or 86% to 91% of all patients with significant positive cultures [113,232], received appropriate therapy. The lack of difference between bacteraemic and non-bacteraemic patients with

sepsis syndrome could therefore be because the deleterious effect of bacteraemia is largely neutralised by effective antibiotics.

There does however seem to be room for further improvement in the outcome of sepsis syndrome patients, despite adequate antibiotics. Ziegler et al [266] showed that the human monoclonal IgM antibody HA-1A reduced the mortality rate from 49% to 30% in patients with sepsis syndrome who had Gram-negative bacteraemia, although the mortality rate rose from 40% to 45% [228] in those patients who did not have Gram-negative bacteraemia. The benefit was greatest in patients who had Gram-negative bacteraemia and shock. No benefit was reported in the group of patients who had Gram-negative infection (defined by culture from the site of infection) but no bacteraemia. There is some doubt about how exactly HA-1A might work [15,16], but the demonstration that it is only active in patients with Gram-negative bacteraemia suggests that these patients are different in an important respect from other sepsis syndrome patients. This is true even after the beneficial effect of antibiotics has been taken into account.

ANTIBIOTICS AND ENDOTOXIN RELEASE.

The finding that antibiotics are of benefit in bacteraemia runs contrary to the idea that antibiotic treatment might lead to release of endotoxin and therefore to worsening of clinical progress [214]. The evidence that significant endotoxin release happens *in vivo* is however weak.

In brucellosis, treatment with tetracycline was followed 8-12 hrs later in half of the patients by a spike of fever, sometimes accompanied by hypotension [220]. This was not however regarded as a serious toxic complication, and was in contrast to the "remarkable" immediate and lasting benefit of treatment. There are anecdotal reports of worsening of typhoid after chloramphenicol treatment [97] but such events have not occurred frequently enough in large series to deserve consideration in a recent review of typhoid treatment [96]. Both *Salmonella typhi* and *Brucella melitensis* are pathogens which multiply within macrophages, and which might therefore trigger macrophage responses in many ways. Chloramphenicol and tetracycline are both bacteriostatic antibiotics, and would therefore not be expected to cause endotoxin release [212].

Rabbit experiments have shown an increase in the ratio of endotoxin to bacteria in the blood following therapy with moxalactam [212] or gentamicin [214]. In these experiments bacteraemia was induced by injecting 20 ml of overnight broth culture of *E. coli*, with mucin, into the peritoneum. The reported rise in endotoxin:bacteria ratio in the treated animals can be explained largely by the fact that antibiotic caused a reduction in the number of viable bacteria in the blood, rather than any marked difference in the amount of endotoxin in the blood. The endotoxin level did continue to rise after antibiotic treatment, but was no greater in the treated animals than in the controls. Endotoxin may well have continued to be absorbed from the very large inoculum (10^9-10^{10} c.f.u.) in the peritoneum.

Recent studies in humans have clearly shown in meningococcal

infection that endotoxin levels in the blood decline consistently and rapidly following the introduction of antibiotic therapy [33]. Rising levels of endotoxaemia have been observed during the first few hours of antibiotic therapy in some patients with septic shock [54] or Gram-negative bacteraemia [213], but these rising levels have not been consistently associated with poor outcome.

HOW DOES BACTERAEMIA LEAD TO DISEASE?

The triggering of cytokine release by endotoxin derived from the invading organisms is the best-known mechanism by which bacterial invasion may lead to clinical sepsis syndrome [83]. This possibility is supported by a vast body of knowledge of the effects of endotoxin. It is supported in the case of meningococcal infection by the demonstration that the LOS level is related to the severity and outcome of disease [33].

Endotoxin in the blood may be absorbed or "translocated" from the gut [242]. This is presumably the mechanism which leads to endotoxaemia in Gram-positive infection [213,241], but begs the question of how bacterial invasion leads to endotoxin translocation. Mechanisms other than the effects of endotoxin must be involved in sepsis caused by Gram-positive infection, and may well be involved in Gram-negative infections also.

A group of protein toxins produced by *Staphylococcus aureus*, *Streptococcus pyogenes* and some other organisms are now recognised as "superantigens" which can interact with the V_β element of the T-cell

receptor and MHC class II antigens on monocytes [93,206]. This stimulates the release of TNFα and IL-1, and can produce systemic illness in animal models. Toxic Shock Syndrome is a well-recognised manifestation of this effect in humans and would in many cases satisfy the clinical criteria for sepsis syndrome.

Other bacterial components are able to induce the features of sepsis syndrome, though with relatively low specific activity. Cell walls of Gram-positive bacteria are pyrogenic [199], and low molecular weight peptidoglycan is probably the active component. Muramyl Dipeptide (MDP) is a synthetic molecule which appears to be the minimum structure required to mimic the effect of low-molecular-weight peptidoglycan, and its activity has been studied in some detail [59]. MDP induced the production of endogenous pyrogen (interleukin-1) from rabbit white blood cells or human mononuclear cells *in vitro*. The action of MDP did not appear to be due to endotoxin contamination of the material, since LAL assays were negative, and cross-tolerance experiments suggest that the mechanism of action of MDP is different from that of endotoxin.

Neither "superantigen" protein toxins nor peptidoglycan fragments can be readily detected in clinical samples. The importance of these factors in sepsis syndrome has therefore not been explored, but this does not mean that they are unimportant.

ENDOTOXAEMIA IN THE ABSENCE OF BACTERAEMIA

Endotoxin assays based on Limulus Amoebocyte Lysate (LAL) were originally developed as a way of rapidly detecting the presence of Gram-negative bacteria, and positive results in the absence of bacteria were initially regarded as false-positives [226]. More recently it has been noted that endotoxaemia is an independent prognostic indicator, and indeed a better indicator for the development of shock [241] or death [54] than is bacteraemia.

Absorption of endotoxin from the gut

The commensal bacteria of the gut can be thought of as a large pool of endotoxin which may represent an important threat to the host. Endotoxin does not seem to be absorbed in health [242], but may be absorbed in disease, for example in patients undergoing surgery for ulcerative colitis [179]. Experimental intestinal ischaemia leads to absorption of endotoxin, via the portal vein [181] or lymphatics [171]. Endotoxaemia itself is a cause of increased gut permeability [175] leading to the absorption of whole bacteria into the lymphatic system [56].

The significance of endotoxin from the gut has been clearly established in heatstroke. Experimental heatstroke in monkeys causes endotoxaemia which can be prevented by pretreatment with oral kanamycin [80]. Prevention of endotoxaemia gives some protection against the cardiovascular effects of heat-stroke. Pretreatment with equine hyperimmune anti-LPS plasma also reduces endotoxaemia and

prolongs survival [79].

ENDOTOXAEMIA IN SEPSIS SYNDROME

It is remarkable, in view of the central role which endotoxin is thought to play in sepsis syndrome [83], how few of the large studies of sepsis syndrome have reported the results of endotoxin assays. This is a reflection of the major practical difficulties in actually performing the assays [45], particularly in the multi-centre studies which have been required to collect large numbers of sepsis syndrome patients. Danner et al [54] measured endotoxaemia in septic shock and showed that it was associated with a poor prognosis. They also found that 57 of 100 patients with septic shock did not have endotoxaemia, despite performing an average of 9.6 endotoxin assays per patient. Either the assays were missing transient episodes of endotoxaemia, or some other mechanism is involved in these patients. This implies that although endotoxaemia is important it cannot be confidently regarded as the only cause of septic shock. This problem may be clarified by the concept of a systemic inflammatory response syndrome [25], which does not assume an infective etiology.

Which endotoxins are involved in sepsis syndrome?

There is some uncertainty over exactly which LPS structures are relevant in clinical sepsis. If Gram-negative blood culture isolates are the major source then the range of LPS from such organisms must be considered. If however LPS is largely derived from organisms in the gut then it is less clear which organisms are

relevant. The gut flora in health is complex and variable. *Bacteroides* out-number the enterobacteriaceae in the colon by approximately 1000 fold [62], so that although *Bacteroides* LPS is 100- to 1000-fold less endotoxic then enterobacterial LPS [131] it may contribute significantly to gut-derived endotoxicity. *E. coli* is the dominant Gram-negative aerobe in the colon of most healthy volunteers, but other enterobacteriaceae dominate in some individuals, particularly after antibiotic treatment [128]. The question of which LPS(s) are important in sepsis becomes crucially important when one goes on to think about antibody treatment aimed at LPS.

CROSS-REACTIVE ANTI-LPS ANTIBODIES.

Several new forms of treatment aimed at blocking the effects of endotoxin are being developed, together with treatments aimed more broadly at blocking the events responsible for sepsis syndrome and septic shock [46,53,162].

Among the various possibilities are antibodies to LPS, both type-specific [53] and cross-reactive. Anti-LPS antibodies have the theoretical advantage of acting at the earliest point in the pathological cascade, but will be useful only in those cases where LPS is a key mediator.

The idea that cross-reactive antibodies to LPS might be useful may be supported by five strands of evidence.

1. Evidence that there are common structures in LPS.
2. Evidence that antibodies to these structures exist, or can be produced.
3. Evidence that the presence of such antibodies prior to sepsis is associated with a good prognosis.
4. Animal experiments which show that such antibodies can be beneficial in experimental sepsis, and *in vitro* experiments which show that the antibodies have effects on the mechanisms of LPS toxicity.
5. Clinical trials of potential therapeutic antibodies in humans with clinical sepsis.

COMMON STRUCTURES IN LPS

The structure of LPS has been reviewed in the first part of this chapter, and it is clear that there are structures common to LPS from many bacterial species, particularly in the inner core and lipid A region. The terminal KDO residue in particular may be an accessible common epitope present in smooth LPS from enterobacteria and *Pseudomonas* [130].

EVIDENCE THAT CROSS-REACTIVE ANTIBODIES TO LPS EXIST

Problems in demonstrating cross-reactive antibodies to LPS

There is a lack of agreement about what constitutes a cross-reactive antibody, and how to demonstrate its presence. In polyclonal sera a major problem is in distinguishing cross-reactivity from the coincidental presence of several antibody specificities. This problem is avoided with MAbs, but difficulties remain in distinguishing specific from non-specific binding, and in determining what level of activity in any assay can be regarded as biologically significant.

Most reports have used assays which simply detect binding of antibody to immobilised antigen. ELISA is now the most popular approach, having replaced the former standard of passive haemagglutination. Binding assays give results which vary according to the way the LPS is immobilised [94,210], and can give false-positive results with

lipid A [5]. The ideal must be to present the antigen in a condition which is "physiological", but that is a currently a subjective judgment. Workers at Borstel have championed the use of passive haemolysis [29]. In this assay the LPS is inserted into a red-cell membrane (which is thought to result in a physiological conformation) and antibody is detected by triggering haemolysis, which is reasoned to be biologically significant.

Absorption and competition have been widely used to demonstrate binding to particular antigens, but this can add to the problems. Absorption or competition with whole bacteria introduces a vast array of new antigens, while absorption or competition with apparently pure materials such as lipid A may also give non-specific results [31]. In more recent studies of the specificity of MAbs antibody competition [187] and competition with synthetic partial structures [32,122,130] have allowed great precision in analysing the specificity of MAbs.

Immunoblotting is useful for demonstrating binding to particular molecular species separated by electrophoresis, though the effect on antigenicity of binding to nitrocellulose is not clear. The drawbacks with this technique are that it is cumbersome, relatively insensitive, and non-quantitative. An alternative and potentially quantitative way of demonstrating binding to particular molecular species is to fractionate the LPS before demonstrating binding [182].

Cross-reactive antibodies as a response to immunisation

The possibility of cross reactive anti-LPS antibodies was initially inferred from animal protection studies [40,148] using antisera to rough mutants of *Salmonella* and *E. coli*.

Initial attempts to demonstrate cross-reactive antibodies in these sera were negative [229]. Subsequently antisera to *Salmonella* lipid A was shown to react with other glucosamine-containing lipid A preparations, but not with Re LPS. Antisera to lipid A and antisera to Re LPS did react with some heterologous smooth LPS or whole bacteria, but this was largely due to the presence of heterologous antibodies in the immune sera which were thought to represent a non-specific mitogenic response to the immunisation. The strongest evidence for presence of an anti-Re cross-reactive antibody in these sera was the demonstration that immunofluorescence with heterologous whole bacteria could be reduced by absorption of the serum with Re LPS [105].

The development of ELISA allowed more sensitive detection of antibody and led to a claim that cross-reactive antibodies could be demonstrated [17]. However, further work by the same researchers produced a more specific ELISA employing LPS complexed with high-density lipoprotein (HDL) which failed to demonstrate cross-reactive antibodies in the sera of humans or rabbits immunised with *E. coli* J5 [14,17].

One of the difficulties encountered in demonstrating cross-reactive

antibodies in J5 antisera was the relatively large amount of J5-specific antibody [17]. An elegant solution to this problem is to affinity purify the cross-reactive antibody with a different rough LPS. This approach has been applied to serum from a J5-immunised calf [237]. The resulting IgG did bind to LPS from *E. coli*, *Salmonella*, *Shigella*, *Pseudomonas aeruginosa* and *Serratia*, and to *Salmonella* lipid A in a simple ELISA assay [238]. However, in view of the possibility of non-specific binding in this type of ELISA [94], and the absence of any comparison with positive-control sera, these results must be treated with caution.

Naturally occurring cross-reactive antibodies in humans

Naturally occurring cross-reactive antibodies may be qualitatively different from those produced by immunisation with rough bacteria or LPS, since naturally occurring antibodies may be produced in response to the core region of whole LPS. Thus the highly specific antibodies to J5, for example may not be encountered in sera from unimmunised individuals, and assays with rough LPS may detect truly cross-reactive antibody in these individuals. This view is supported by the finding that the specific activity of affinity purified anti-Re IgG from rabbits immunised with *S. minnesota* R595 was approximately 10 times higher than affinity-purified anti-Re IgG from unimmunised humans [224], and by absorption studies discussed below [211].

Antibodies to lipid A can be detected in human sera, particularly in those from individuals who have had Gram-negative infection [140]. Lipid A has been shown to inhibit the binding of naturally-occurring

antibodies in human sera to rough LPS [36], but the ability of these antibodies to cross-react with whole bacteria or smooth LPS has not been defined.

Barclay and colleagues have studied the specificity of naturally occurring antibodies to endotoxin (reviewed by Barclay [9]). They developed an ELISA using LPS complexed with polymyxin which gave more reliable results than standard ELISA. This assay was also able to detect some naturally-occurring antibodies to J5 LPS which were not detected by conventional LPS ELISA, perhaps because LPS complexed with polymyxin was locked into the conformation encountered naturally in the host [210]. (Polymyxin has been shown to inhibit the binding of some anti-lipid A MAbs to LPS [65,163,189], and may therefore block some epitopes, but this does not seem to be a practical problem.) Using this assay Barclay and Scott [10] studied IgG antibodies to a variety of rough and smooth LPS chemotypes in 763 blood donor sera. They found a strong correlation between antibody to *Salmonella* Rc LPS and antibody to Ra, Rb or smooth LPS. Antibody to Re LPS associated with antibody to Ra and smooth LPS in only about half of the sera. They concluded that Rc or Rb LPS would be the best antigen to use to screen blood donor sera for cross-reactive anti-LPS antibodies. This assay seems to be better at detecting cross-reactive antibodies than simple ELISA with J5 or R595 LPS, which both seem to detect more chemotype-specific antibody [225,244].

A cocktail of four rough LPSsof Rc or greater-sized chemotype (*E. coli* K12, *Klebsiella pneumoniae* M10b, *P. aeruginosa* PAC605, *Salmonella typhimurium* 878) complexed with polymyxin was then used to

screen blood donor sera. IgG prepared from sera with high levels of antibody to this cocktail reacted with each of the individual components of the cocktail, and with a range of other *Salmonella* and *E. coli* LPS preparations. In three sera studied in detail most of the measured antibody could be removed by absorption with *Salmonella* bacteria expressing Re (2 cases) or Re (one case) LPS [211]. This was compatible with the concept of common cross-reactive antibodies directed against the structures present in Re LPS, and confirmed that the polymyxin-LPS-core cocktail assay was a potentially useful way of screening blood donor sera.

Using the polymyxin-LPS-core cocktail assay, Jackson et al [100] measured the levels af anti-LPS-core antibody in children, and found predominantly IgG antibody. The antibody was deficient in premature infants, but was present (as maternal antibody) in term infants and normal children, and reached adult levels by about 15 years of age. Law and Marks [126], using an ELISA with J5 LPS as antigen, obtained broadly similar results except that they found predominantly IgM. This difference could be explained by difference in the relative potency of the IgG and IgM conjugates used.

Nys et al [174], using an ELISA with R595 (Re) LPS, found that the anti-Re IgG in blood donors was predominantly of IgG2 subclass, while the IgG4 sublclass was found only in patients with bacteraemia.

Levels of antibody to LPS-core antigens have been found to be greater in normal individuals from some third-world locations than in normal individuals from the USA [225]. These differences are related to,

but not completely explained by, differences in total immunoglobulin levels.

Cross-reactive Monoclonal antibodies (MAbs)

There have been numerous attempts to produce cross-reactive MAbs to endotoxin. Early reports of cross-reactivity were based on ELISA [63,65,163,203,230] which may detect non-specific binding, as discussed above. Binding to heterologous heat killed whole bacteria but not LPS was noted in several cases [8,163,189].

The range of organisms used to demonstrate cross-reactivity varied enormously. Nelles et al [163] used a total of 32 isolates, including *Acinetobacter*, *Pseudomonas*, *Klebsiella* and *Serratia* as well as clinical isolates of *E. coli*, although the degree of binding detected with many of these organisms was much less than that with the J5 immunising strain. Teng et al [230] used an even wider range of genera. Neither of these groups showed results for a negative control Gram-positive organism. Erich et al [65] used one *Shigella*, two *Salmonella* and one *E. coli* while Salles et al [203] used four *E. coli* and single strains of *Klebsiella* and *Serratia*. Both of these latter groups used enteropathogenic strains of *E. coli* rather than blood-culture strains.

Many of the MAbs studied in detail recently [32,55,122,130,187] have been found to bind to epitopes found only on rough mutant LPS or isolated lipid A, or have been found to react with only one or two core types [41,133,170,245]. Two MAbs with well-defined epitopes

(8A1 and clone 20) do appear to be cross-reactive however, as do a number of other MAbs with less well defined epitopes.

The murine IgG MAb 8A1 has been shown to recognise the bisphosphorylated backbone of lipid A [122], and is reported to be cross-reactive in solid-phase radioimmunoassay (RIA) (with heat-killed bacteria) and immunoblotting with *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *Salmonella minnesota* but not *Serratia marcescens* [21]. Clone 20, a murine IgG, reacts with the terminal KDO residue [32], and has been found to be cross-reactive in ELISA with smooth LPS from one strain of *K. pneumoniae* and with heat-killed cells of one strain of *P. aeruginosa*, [5]. The data on clone 20 should be treated with caution however as it was also reported to bind in ELISA to lipid A despite evidence that its epitope is the terminal KDO.
The murine IgM MAb 8-2C1 [55] has a less well-defined epitope in the KDO-lipid A region, and is said to bind in ELISA to heat-killed *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *Enterobacter cloacae*, *Proteus mirabilis Morganella morganii* and *Serratia marcescens*.

A study of two human anti-LPS-core MAbs found two patterns of cross-reactivity in ELISA, one involving *E. coli*, *K. pneumoniae* and *P. aeruginosa*, and the other involving many enterobacteriaceae but not *P. aeruginosa* [255]. This underlines the need to establish in detail the range of cross-reactivity of particular MAbs.

Studies of the binding of E5, a mouse MAb which has been the subject of two major clinical trials, have recently been published [182,257]. They show binding to lipid A, cross-reactivity with representatives

of six genera of enterobacteriaceae and with *P. aeruginosa*, and binding to both O-substituted and unsubstituted LPS. The methods used to show binding to lipid A were principally ELISA and solid-phase RIA in which plates were coated with LPS dissolved in methanol. This was very similar to the method shown by Heumann et al [94] to produce false cross-reactions, although the E5 studies did include a negative control MAb as evidence of specificity. In addition a LAL antibody-capture assay was used in which LAL was used to detect LPS captured by immobilised E5 MAb.

Peer-reviewed data on the epitope specificity and cross-reactivity of the HA-1A the other MAb which has been the subject of a major clinical trial, is scanty [20,230].

EVIDENCE THAT NATURALLY-OCCURRING ANTIBODIES PREVENT SEPSIS

Several workers have shown a relationship between naturally-occurring antibodies and the course of endotoxin-related disease. McCabe et al [152,268] showed that the presence of high titres of antibody to Re 595 LPS was associated with a lower incidence of shock and death in Gram-negative bacteraemia. An interesting negative finding in this study was that antibody to enterobacterial common antigen (ECA) was not associated with a favourable outcome, suggesting that the Re 595 result is not merely a reflection of greater previous exposure to Gram-negative infection. Pollack et al [188] similarly showed that antibody to *E. coli* J5 LPS, particularly IgM antibody, was associated with survival in *P. aeruginosa* septicaemia. A similar relationship might be expected in other conditions where endotoxin plays a part.

IgM antibody to *E. coli* J5 LPS has been shown to be associated with a reduced risk of graft-versus-host disease following bone-marrow transplantation [47].

A more complex hypothesis studied by Barclay et al [11] was that cross-reactive antibody would be consumed during endotoxaemia, and that symptoms would occur when the endogenous supply of antibody was no longer sufficient to neutralise the endotoxin. Detailed studies of three patients with septic shock supported this view, showing a drop in antibodies to polymyxin-complexed rough LPSs (ranging from Ra to Re) associated with endotoxaemia. Similar conclusions were reached by Nys et al [172,173] who found that a high or rising level of IgG antibody to R595 (Re) was associated with a favourable outcome in patients in surgical intensive care, while a falling level of anti-Re IgG was predictive of a poor outcome.

ANIMAL EXPERIMENTS AND *IN VITRO* MODELS OF PATHOGENESIS

The concept of cross-reactive antibodies to LPS for the treatment of sepsis was originally based on passive transfer of sera from rabbits immunised with *E. coli* J5 and *Salmonella* Re 595 [34,148]. These effects can be produced by the IgM fraction of serum [147]. In the light of research which has failed to repeat these experiments [88] and failed to find cross-reactive antibodies in these sera [94] the results are difficult, although not impossible [264] to explain. It is certainly clear that the results of animal experiments are extremely complex and subject to many variables, and must therefore be interpreted with caution.

Factors which may produce false-positive results include protective factors in the sera of pre-immune donor animals [87] non-specific B-cell mitogenicity, acute phase reactants [88], and endotoxin contamination of antibody preparations [42,258]. False-negative results (reviewed by Ziegler [264]) may be produced by use of the wrong animal model, excessive challenge inoculum, or unreasonable comparisons between cross-reactive sera and much more active homologous sera.

In animal models some anti-LPS MAbs have been found to be protective [64], while others have not [6,159]. MAbs apparently sharing the same epitope even differed in their protective activity [6], but more detailed analysis revealed fine differences in epitope [32]. If the mechanisms of action of endotoxin were well understood it would be possible to differentiate these types of MAb *in vitro*. If TNF is the key mediator then testing MAbs for inhibition of LPS-induced TNF production *in vitro* may be a useful way of looking for protective MAbs [44]. However it has been shown that one MAb (clone 20, see above) can have protective effects in an animal model without influencing the levels of TNF and IL-6 [218], so that *in vitro* TNF suppression may not be essential for protective activity. Other possible *in vitro* approaches to identifying protective MAbs have been to examine antibody subclass or inhibition of binding by polymyxin [6], or inhibition of *Limulus* activation [154], but none of these has proved useful. Ability of antibodies to initiate complement-mediated bacterial killing (itself a function of antibody class) has been shown to be related to protection in an infection model with O-specific MAbs [176]. This may not be relevant to cross-reactive

MAbs, however, and is unlikely to be a predictor of *in vivo* anti-toxic activity of MAbs.

HUMAN CLINICAL TRIALS

The ultimate test of an antibody treatment must be a clinical trial. Several large and carefully planned trials have been mounted, but as yet none have produced results good enough to support routine clinical use of any anti-LPS antibody treatment.

The first large trial [267] involved a comparison of serum from J5-immunised donors with pre-immune serum from the same donors. These sera were given to patients with a clinical diagnosis of Gram-negative bacteraemia. There was a significant reduction in mortality in the bacteraemic patients, particularly those with shock. A similar comparison between J5-immunised and pre-immune serum (or plasma) as prophylaxis against Gram-negative infection in high-risk surgical patients [13] also showed beneficial effects, reducing the incidence of shock related to Gram-negative infection. (The statistical significance of the findings in this trial has since been questioned by its first author [12].)

Treatment with whole serum or plasma has practical problems [38], including the increasing possibility of HIV transmission. IgG formulated for intravenous administration (IVIG) avoids these problems. Calandra et al [38] compared IVIG prepared from J5-immunised donors with standard IVIG in the treatment of Gram-negative infection and found no difference in outcome. This may indicate that

the beneficial effect of J5 antiserum was in the IgM fraction.

Two IgM MAbs, HA-1A and E5 had by now become available and were entered into clinical trials. The results of the HA-1A trial [266] looked promising, and had met with an enthusiastic initial response [4]. There was a significant reduction in mortality in patients with Gram-negative bacteraemia, particularly when associated with shock, as had been found with J5 antiserum. Overall however there was no improvement in the outcome of the patients treated because of a small (not statistically significant) increase in mortality in the non-bacteraemic patients who received HA-1A [228]. More detailed re-examination of the available data [249] pointed to some inequalities beween the patient and control group (for example there were more patients in the control group who received inadequate antibiotics) and concluded that the value of HA-1A remained unproven. This re-examination also emphasised the lack of understanding of exactly how HA-1A worked and therefore the lack of any assay which could be used in quality control of production batches.

The E5 trials were even less persuasive despite the apparently superior affinity of E5 for LPS [182,257]. It was beneficial in patients with Gram-negative infection, whether bacteraemic or not, only if they were not in septic shock. These beneficial results were not confirmed in a repeat trial ([253], cited by [249]).

Running in parallel with the recent studies of MAbs have been further studies with immunoglobulins derived from blood donors. Dominioni et al [60] gave a total of 1g/Kg of IgG (Sandoglobulin) to 29 severely

ill septic patients and found a reduction in mortality compared to a control group of 33 patients given albumin. Schedel et al [204] studies the effects of a different immunoglobulin preparation (Pentaglobin) which contains IgM and IgA as well as IgG, and which was said to have 8 to 16 fold higher "specific antibody titres to different determinants of lipopolysaccharide" than a random-donor, normal human serum pool" (data attributed to personal communication from BJ Appelmelk). They gave a total of 74.4 g of this preparation to each of 27 patients with septic shock and detectable endotoxaemia, and found only one death compared to 9 deaths in a control group of 28 similar patients given no immunoglobulin. Both of these results could be explained by beneficial effects of naturally-occurring antibodies to LPS, though these antibodies may be a mixture of very specific antibodies rather than cross-reactive antibodies.

The latest twist to this tale comes from a study by Cometta et al [48] which compared standard IVIG to IVIG from donors found to have high levels of antibody to Re595 LPS, and to an albumin placebo. Up to four doses of 0.4 mg/Kg IVIG or albumin were given as prophylaxis to patients at high risk of post-surgical infection, using the same entry criteria as Baumgartner et al [13]. Unfortunately the number of cases of Gram-negative infection or septic shock was too small to evaluate any effect [217]. There was however a reduction in hospital-acquired infection in the group given standard IVIG, but no reduction in the group given selected anti-Re IVIG. The mechanism by which this reduction might occur is not clear, but it does suggest that serum selected for a high level of anti-Re antibody may be lacking in some other important antibody.

AIMS OF THIS THESIS

The story of cross-reactive antibodies to LPS is complex. There is no certainty that antibodies really exist that are cross-reactive enough to be active against the range of LPS found in human sepsis, and there is no very clear demonstration of their efficacy in humans. There is however considerable evidence to suggest that such antibodies may be important in infection, and that beneficial antibodies could be produced (as MAbs) or selected from donor sera.

I established links with a group of researchers active an this field and, within the limits of the techniques and materials available to me, tried to produce some useful results. I have presented in this thesis 5 chapters of results:

Chapter 3: Antibody response in recipients of typhoid vaccine. This was an attempt to determine whether high levels of antibody detected by polymyxin/LPS-core cocktail ELISA could be explained by an association with typhoid vaccination.

Chapter 4: IgG antibody in urine in patients with urinary tract infection. This was an application of the polymyxin/LPS-core cocktail ELISA to the diagnosis of urinary tract infection.

Chapter 5: Studies on the interaction of MAb and human IgG preparations with blood-culture isolates of Gram-negative bacilli and rough mutants of *E. coli*. In this chapter I present the

results of studies done as part of the characterisation of a large panel of MAbs and some human IgG preparations.

Chapter 6: Core-typing of E coli. This work arose from an observation made in the experiments described in chapter 5, that some of the MAbs produced could distinguish between different core types of wild-type *E. coli*. The aim was to determine the frequency of the different core types in clinical isolates of *E. coli*, with the further purpose that this information should be of value in assessing the ability of MAbs to interact with common clinical isolates.

Chapter 7: Studies of some cross-reactive antibodies not specific for LPS-core. The experiments described in this chapter aimed to characterise three non-LPS MAbs which were produced, and also include some experiments with HA-1A.

CHAPTER 2

GENERAL MATERIALS AND METHODS

This chapter contains details of materials and methods used in more than one of the succeeding chapters of results. Materials and methods specific to one chapter are included in that chapter.

BACTERIA AND BACTERIOPHAGE

Many different bacteriophage and bacterial strains, obtained from several different sources, have been used in the work described here. Tables of sources and reference numbers are included in the relevant section of results. There were two main sources of strains, as follows:

1. Microbial Pathogenicity Research Laboratory (MPRL), Department of Medical Microbiology, Edinburgh University. All bacterial strains in this collection are assigned an MPRL reference number and stored in freeze-dried form. Bacteriophages are stored as suspensions in nutrient broth at 4°C saturated with chloroform.

2. Clinical Bacteriology Laboratory, Department of Medical Microbiology, Edinburgh University. All blood-culture isolates from patients in the Royal Infirmary, Edinburgh are kept. Strains are initially stored on nutrient agar slopes and subsequently freeze-dried in batches. Reference numbers for blood-culture strains contain a "b" (eg 8b281). Enteric pathogens from stool cultures are stored on nutrient agar slopes. Urine isolates were kept either freeze dried (in the case of the 6 strains used in chapter 4) or on agar slopes (in the case of the 80 strains used in chapter 6).

Bacterial Culture Media

Nutrient Broth was Gibco nutrient broth, prepared and filter-sterilised at the Blood Transfusion Service Protein Fractionation Centre, Edinburgh.

Solid media used were nutrient agar (Columbia agar base, Oxoid, Basingstoke) and Cysteine Lactose Electrolyte Depleted (CLED) agar (Oxoid), both prepared by the media kitchen of the Department of Medical Microbiology.

Bacterial Culture

All bacteria were cultured at 37°C in air. Broth cultures of between 10 ml and 100 ml were prepared by inoculating colonies from agar media into broth and incubating overnight at 37°C in a Gallenkamp shaking incubator. Larger volumes were seeded with 1% of starter culture prepared as above. Purity of all cultures was checked by streaking-out to single colonies on CLED agar.

MONOCLONAL ANTIBODIES (MABS)

All MAb preparations (except HA-1A) were produced as part of collaboration between Sandoz (Basle) and researchers in Edinburgh. BALB/c mice were immunised with bacteria or LPS immunogens both in Edinburgh and Basle. Several different protocols were used, the details of which are to be published elsewhere. Spleen cells from immunised mice were fused with NS-0 myeloma cells using standard

techniques [114] in the Department of Surgery, Edinburgh University, and by Sandoz. In Edinburgh, MAbs of interest were selected by Dr GR Barclay of the Blood Transfusion Service on the basis of reactivity with a range of rough LPS in polymyxin-LPS ELISA. The antigens used for screening included the immunogen(s) that had been used in that particular mouse together with other LPS selected to complement the immunogen(s). The clones produced in Basel were initially screened for reactivity with bacteria in ELISA. MAbs were then further characterised by LPS/polymyxin ELISA with a wider range of LPS and lipid A preparations, mainly from *E. coli* and *Salmonella* species. The LPS preparations used were obtained from commercial sources (Sigma and List Biologicals) or were produced in MPRL or by Dr H Brade at the Forschungsinstitut, Borstel, Germany, as listed in table 2.1.

MAbs preparations used in this thesis were either supernatants of hybridoma cell cultures (in RPMI 1640 medium supplemented with 5% fetal calf serum, antibody concentration 10-50 µg/ml) or purified preparations containing 5-10 mg/ml of antibody.

Table 2.1 LPS preparations used to characterise monoclonal antibodies

Species	Number	Chemotype	Source:
Salmonella minnesota:	(R595)	lipid A	List
"	R595	Re	List,Sigma
"	R7	Rd	Sigma
"	R5	Rc	List
"	R345	Rb	List
"	R60	Ra	List
"		Smooth	List
S.typhimurium	1102	Re	MPRL
"	SL1181	Re	Sigma
"	1032	Rd	MPRL
"	SL684	Rc	Sigma
"	878	Rc	MPRL
"	119	Rb	MPRL
"	TV119	Ra	Sigma
"		Smooth	Sigma
"	1542	Smooth	MPRL
S. abortus-equi		Smooth	Sigma
S. typhi		Smooth	Sigma
S. enteritidis		Smooth	Sigma
E. coli	(D31m4)	lipid A	List
"	K12 D31m4	Re	List
"	F515	Re	Borstel
"	F853	Rd	Sigma
"	J5	Rc'	Sigma,List
"	K12 mm294	Ra	List
"	R1 HF 4704	Ra	MPRL,Borstel
"	R2 F576	Ra	Borstel
"	R2 EH100	Ra	Sigma,MPRL
"	R3 F653[d]	Ra	Borstel,MPRL
"	R4 F2513	Ra	Borstel,MPRL
"	C62	Ra'	MPRL
"	Rf Tn10	Ra	MPRL
"	O:2	Smooth	MPRL
"	O:4	Smooth	MPRL
"	O:6 K5	Smooth	MPRL
"	O:12	Smooth	MPRL
"	O:15	Smooth	MPRL
"	O:16	Smooth	MPRL
"	O:18 K1	Smooth	MPRL
"	O:18 K-	Smooth	MPRL
"	O:55 B5	Smooth	Sigma
"	O:75	Smooth	MPRL
"	O:86	Smooth	MPRL
"	O:111 B4	Smooth	List,Sigma
"	O:127 B8	Smooth	Sigma
Pseudomonas aeruginosa	Habs-1	Smooth	MPRL
"	Habs-10	Smooth	Sigma
"	C605	Rb	MPRL
Vibrio cholerae	O:1 inaba	Smooth	Sigma
Serratia marcescens		Smooth	Sigma
Klebsiella aerogenes	M10b	Rb	MPRL
Shigella flexneri		Smooth	Sigma
"		Re	Sigma

ENZYME-LINKED IMMUNOSORBENT ASSAYS (ELISA)

Buffers

Phosphate buffered saline (PBS) was 50 mM phosphate, 0.15 M sodium chloride, pH 7.4.

Coating buffer was 0.05 M sodium carbonate-bicarbonate pH 9.6 with 0.05% sodium azide.

Wash buffer was PBS with 0.05% sodium azide and 0.1% Tween-20.

Post-coating buffer was PBS containing 5% bovine serum albumin and 0.05% sodium azide.

ELISA diluent was PBS with 0.05% Tween-20 , 0.5% bovine serum albumin, 4% polyethylene glycol 6000 (Sigma, formerly called polyethylene glycol 8000), and 0.05% sodium azide. For work with monoclonal antibodies the albumin was omitted.

Polymyxin-LPS ELISA plates

These plates were prepared by Linda Milne in the Microbial Antibodies Laboratory, Blood Transfusion Service. The LPS used was prepared by Frances McLoughlin in the Microbial Pathogenicity Research Laboratory, except where attributed to a commercial source.

Smooth LPS were prepared by the aqueous phenol method of Westphal and

Lüderitz [254], and rough LPS were prepared by the aqueous phenol, chloroform, petroleum ether method of Galanos et al [76] incorporating the diethyl ether, acetone precipitation of LPS described by Qureshi et al [191].

LPS was made up to a concentration of 0.2 mM in pyrogen-free sterile water and added to an equal volume of 0.4 mM polymyxin B (Sigma) then mixed with continuous stirring for 30 minutes at room temperature to allow LPS-polymyxin complexes to form, dialysed overnight at room temperature to remove excess unbound polymyxin [161], and stored at -20°C. To coat plates, complexes were sonicated for 30 seconds, diluted 1 in 50 in coating buffer, and dispensed (100 µl per well) into Nunc polysorb plates. The plates were incubated overnight at room temperature, then washed four times with wash buffer. Post-coating buffer (100µl per well) was then added and incubated overnight at room temperature, and washed as above. Plates were wrapped in plastic film and stored at -20°C.

Polymyxin-LPS-core cocktail ELISA plates

Polymyxin-LPS-core cocktail ELISA plates were made up as above using equal molar quantities of rough LPS from *E. coli* K12, *Klebsiella pneumoniae* M10b, *Pseudomonas aeruginosa* PAC605, *Salmonella typhimurium* 878.

Serum anti-LPS-core assay

ELISAs on serum using polymyxin-LPS-core cocktail plates were performed using a 1:400 dilution of serum in ELISA diluent with 0.5% bovine serum albumin. Dilute serum (100 µl) was added per well in triplicate, incubated at 37°C for 60 min, and washed five times. Then 100 µl of urease-conjugated sheep-anti-human-IgG (Sera-lab, diluted 1 in 500 in ELISA diluent with 0.5% bovine serum albumin) was added, incubated for a further 60 min at 37°C, then washed three times and rinsed five time in distilled water. Urease substrate (100 µl: Sera-lab) was added and left at room temperature for 60 min, and OD read at 590 nm. Blank readings (obtained with wells which had no LPS-polymyxin complexes added but which had been post-coated) were subtracted. Results were expressed as percentage of a known positive control (chapter 3), or as median units where 100 was the median value determined from assays on 1,000 blood donor sera (chapter 4).

POLYACRYLAMIDE GEL ELECTROPHORESIS AND IMMUNOBLOTTING

Sample buffer (single strength) was 0.0625 M Tris/HCl, 2% sodium dodecyl sulphate, 10% glycerol, 1% 2-mercaptoethanol, 0.001% bromophenol blue. Double-strength sample buffer was made up with concentrations of all constituents doubled to be mixed with an equal volume of an aqueous solution of LPS or protein.

Preparation of proteinase K digests [95]

Bacteria were grown up overnight in nutrient broth in a shaking incubator. Broth was then centrifuged at 3000 g (5000 rpm) for 10 minutes in a bench centrifuge. The pellet was resuspended in 10 ml PBS, centrifuged again and resuspended in PBS, and the concentration adjusted to give an optical density (OD) of 0.5 - 0.6 at 525 nm. Aliquots of 1.5 ml were placed in screw-capped Eppendorf tubes and centrifuged for 5 minutes at 10,000 g (13000 rpm) in an MSE Microcentaur centrifuge. Supernatant was poured off and the pellet resuspended in 50 µl of single-strength sample buffer. This suspension was then heated to 100°C for 10 minutes in a boiling water bath. After allowing the suspension to cool, 10 µl of a 2.5 mg/ml solution of proteinase K (Protease type XI; Sigma) was added, and the mixture incubated at 60°C for 1 hour. The preparation was then stored at -20°C until required.

For the production of larger quantities of proteinase K digest the method above was scaled up. A 15 ml volume of bacterial suspension prepared as above was centrifuged at 5000 rpm in the bench centrifuge in a universal container, resuspended in 500 µl of sample buffer, and then transferred to a screw-capped Eppendorf tube for heating and digestion with 100 µl of proteinase K solution. These preparations were then divided into 100µl aliquots for storage.

Polyacrylamide gel electrophoresis and immunoblotting

Proteinase K preparations (20µl aliquots) or LPS (10 µg in 20µl sample buffer) were separated on 14% polyacrylamide gel [124] omitting SDS from stacking and separating buffers. Protein preparations (25µg) were separated on polyaccylamide gel with SDS. Gels were then silver stained (for LPS) [95], or stained with Coomassie blue (for protein) [190] or blotted [233] to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany: 0.2 µm pore size). Blots were blocked with 3% wt/vol gelatin in Tris buffered saline pH7.5 (TBS), incubated for 3 h in a dilution of antibody in 1% gelatin in TBS. Unless otherwise stated the dilutions used were 1 in 10 (1-5 µg/ml) for MAb supplied as culture supernatants or 1 in 500 (10-20 µg/ml) for purified MAb preparations. This was followed in the case of murine IgG MAbs by 1 hour incubation with horseradish peroxidase anti-mouse IgG conjugate (ICN, diluted 1 in 1000), and developed with HRP colour reagent (Bio-Rad). For IgA and IgM MAbs anti IgG/M/A conjugate (Zymed, diluted 1 in 500) was used. For human antibodies, anti-human-IgG (Miles, diluted 1 in 500) or anti-human-IgM (ICN, diluted 1 in 500) conjugates were used as appropriate.

CHAPTER 3

ANTIBODY RESPONSES IN RECIPIENTS OF TYPHOID VACCINE

INTRODUCTION

During the process of screening of blood donor sera for IgG antibody to LPS core with polymyxin/LPS-core ELISA [9,211] a subjective impression was gained by those involved that there was an association between high levels of antibody and history of travel to areas where malaria is endemic. (Information about travel to such areas is routinely recorded by the transfusion service because of the risk of malaria transmission, and blood from these individuals is not used therapeutically.) One possible explanation for this would be that the antibody was increased following typhoid vaccination. This was an interesting hypothesis because it could lead to the use of typhoid vaccine to boost anti-LPS-core antibody levels in blood donors or in individuals who might in future be at risk of endotoxaemia. The use of typhoid vaccine for this purpose would have the major practical advantage that it is already approved for use in humans, and has an accepted safety record. I therefore set out to investigate this possibility by looking at antibody levels in recipients of typhoid vaccine.

MATERIALS AND METHODS

Sera

Sera were obtained from two groups of vaccine recipients. The first group consisted of 11 students who each received two doses of Wellcome monovalent killed typhoid vaccine. Sets of three sera were collected from each. In each case serum was obtained a) on the day

of the first dose of vaccine; b) on the day of the second dose of vaccine (around 4 weeks after the first dose); and c) 2-4 weeks after the second dose of vaccine. All sera were obtained before any foreign travel. The second group were 9 soldiers on an induction course. They each received two doses of typhoid and paratyphoid A and B (TAB), together with tetanus toxoid. This vaccine was prepared for the army at Porton Down. Due to a misunderstanding over the desired timing, these samples were obtained a) 19 days after the first dose; b) 1 day after the second dose; and c) 10 weeks after the second dose.

Widal assays

These tests were carried out using Wellcome stained salmonella suspensions SS01 (*Salmonella typhi*-H), SS09 (*Salmonella typhi*-O) and SS13 (*Salmonella paratyphi* C-O). Heat-inactivated sera were diluted 1 in 20 in saline, and serial doubling dilutions made to a maximum of 1 in 640. The appropriate antigen dilution (10µl) was added to 250 µl of serum dilution in a narrow glass tube. This dilution is equivalent to the recommended 1 drop (40µl) in 1 ml of serum dilution recommended by the manufacturer. A control tube with no serum was prepared for each antigen. Tubes were mixed and heated at 50°C for two hours for the O antigens, or four hours for the H antigens. The highest dilution of serum giving agglutination of the antigen was recorded as the titre of antibody.

All sera were assayed by the Widal method for antibody to *Salmonella typhi* O and H antigen, and *S. paratyphi*-C O antigen. IgG antibody to polymyxin/LPS-core cocktail was measured by ELISA. Sera from 8 individuals were then tested for antibody to a range of 23 individual LPS preparations in LPS-polymyxin ELISA.

Among the students (figure 3.2) there was a rise in antibody to *S. typhi* H antigen in all cases, and a rise in antibody to *S. typhi* O antigen in all but one. The individual who had no rise in antibody to *S. typhi* O was the only one to show a rise in antibody to LPS-core. None of the students had a rise in antibody to *S. paratyphi*-C O antigen.

Among the soldiers (figure 3.1) the initial *S. typhi* and *S. paratyphi*-C antibody titres were similar to, or lower than, those seen in the students. This suggested that the initial samples did represent pre-immunisation antibody levels, despite the delay in their collection. A rise in antibody to *S. typhi* H antigen was observed in all but one of the soldiers, and a rise in antibody to *S. typhi* O was observed in all but two. (In one individual there was no rise in antibody to *S. typhi* O or H antigens.) A small rise in antibody to LPS-core was observed in two soldiers. One of these two had a large rise in antibody to *S. typhi* O antigen, while the second had a small rise(from 1:20 to 1:40) in this antibody. None of the soldiers had a rise in antibody to *S. paratyphi*-C O antigen.

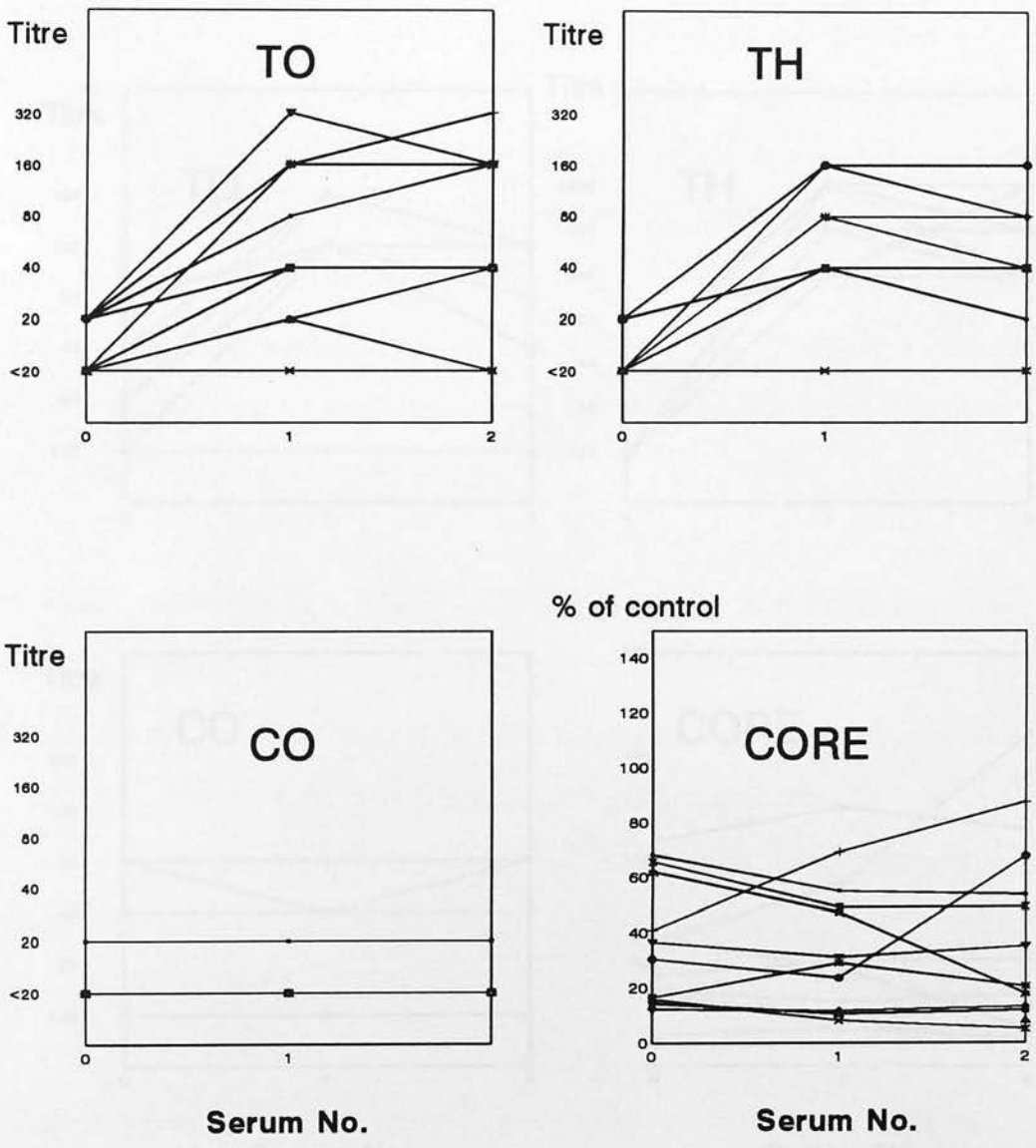


Figure 3.1 Antibodies in sera from 9 army recruits immunised with killed typhoid/paratyphoid (TAB) vaccine and tetanus toxoid. Antigens are as follows: TO = typhoid O; TH = typhoid H; CO = paratyphoid-C O (all Widal assays); CORE = polymyxin-LPS-core cocktail IgG ELISA (expressed as % of control). Serum samples were taken as follows: 0 = 19 days after first dose, 1 = 1 day after second dose, 2 = 10 weeks after second dose.

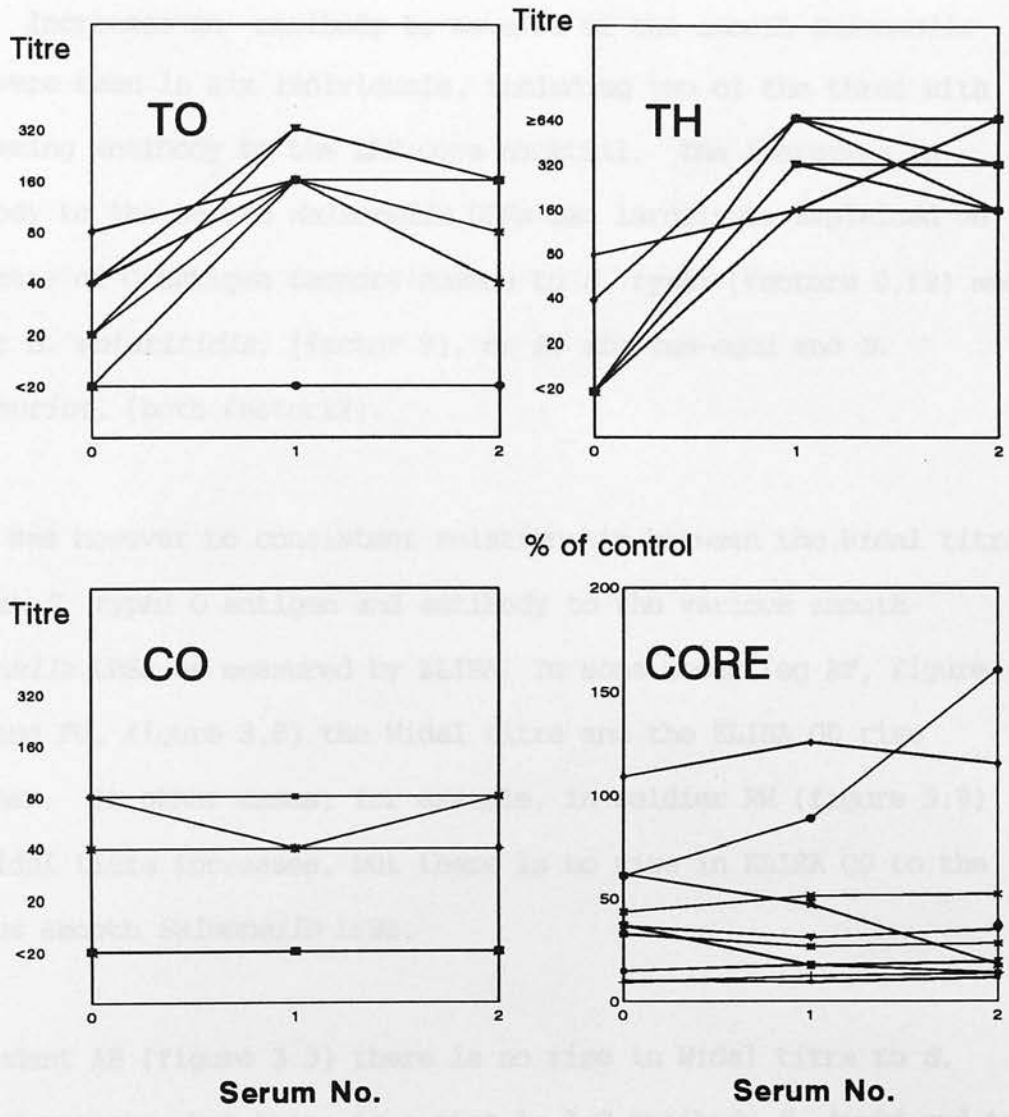


Figure 3.2 Antibodies in sera from 11 students immunised with monovalent killed typhoid vaccine. Antigens are as follows: TO = typhoid O; TH = typhoid H; CO = paratyhpoid-C O (all Widal assays); CORE = polymyxin-LPS-core cocktail IgG ELISA (expressed as % of control). Serum samples were taken as follows: 0 = before first dose, 1 = before second dose, 2 = 2 - 4 weeks after second dose.

Sera from 8 individuals were selected for measurement of antibody to individual LPS chemotypes (figures 3.3 - 3.10). These included the three individuals with a rise in LPS-core antibody (figures 3.3, 3.4, 3.9). Increases in antibody to several of the smooth *Salmonella* LPSs were seen in six individuals, including two of the three with increasing antibody to the LPS-core cocktail. The increases in antibody to the smooth *Salmonella* LPSs can largely be explained on the basis of O antigen factors common to *S. typhi* (factors 9,12) and either *S. enteritidis*, (factor 9), or *S. abortus-equi* and *S. typhimurium*, (both factor12).

There was however no consistent relationship between the Widal titre against *S. typhi* O antigen and antibody to the various smooth *Salmonella* LPSs as measured by ELISA. In some cases (eg AT, figure 3.7, and PU, figure 3.8) the Widal titre and the ELISA OD rise together. In other cases, for example, in soldier RW (figure 3.9) the Widal titre increases, but there is no rise in ELISA OD to the various smooth *Salmonella* LPSs.

In student AB (figure 3.3) there is no rise in Widal titre to *S. typhi* O antigen, but there is a rise in IgG antibody *S. typhi* and to the cross-reacting *Salmonella* serotypes (*S. abortus-equi* and *S. enteritidis* but not *S. minnesota*). This individual also showed a marked increase in antibody to the polymyxin-LPS-core cocktail. It is possible that the antibody measured with the smooth *Salmonella* LPSs is in fact anti-core antibody. This seems unlikely, however, since there is no increase seen with the *Salmonella* Ra LPSs, and the antibody to smooth *Salmonella* LPSs is restricted to those known to

cross-react with *S. typhi*.

In the three individuals with a rising antibody to polymyxin-LPS-core cocktail (soldiers YG (figure 3.4), and RW (figure 3.9) and student AB (figure 3.3)) there is no detectable rise in antibody to the individual rough LPSs that go to make up the cocktail. The only rough LPS which detects a rise in antibody is *E. coli* R1, which is not part of the cocktail. Antibody to *E. coli* R1 also rises in soldier US (figure 3.6) despite a falling anti-LPS-core cocktail IgG in this case.

No significant rises were observed in the rough LPSs that go to make up the LPS-core cocktail, nor was there any consistent increase in antibody to Ra core of *Salmonella*. There was therefore no consistent rise in cross-reactive antibody to LPS-core.

Table 3.1

Abbreviations for LPS preparations used in figures 3.3 - 3.10

Abbreviation	Species	LPS Prepared by
SABORTUS	*Salmonella abortus-equi*	Sigma
SENTRDSIG	*Salmonella enteritidis*	Sigma
SMWT	*Salmonella minnesota*	MPRL
SMWTSIG	*Salmonella minnesota*	Sigma
STWTSIG	*Salmonella typhimurium*	Sigma
STYPHI	*Salmonella typhi*	Sigma
SERMARSIG	*Serratia marcescens*	Sigma
ECK12	*E. coli* K12	List
ECO18R	*E. coli* 18 rough mutant	MPRL
ECR1BR	*E. coli* R1 HF4704	Borstel
ECR2BR	*E. coli* R2 F576	Borstel
ECR3BR	*E. coli* R3 F653	Borstel
ECR4BR	*E. coli* R4 F 2513	Borstel
SMRA	*Salmonella minnesota* R60 (Ra)	List
STRA	*Salmonella typhimuirium* TV119 (Ra)	Sigma
KAM10B	*Klebsiella aerogenes* M10b	MPRL
PAC605	*Pseudomonas aeruginosa* PAC 605	MPRL
SMRC	*Salmonella minnesota* R5 (Rc)	List
STRC	*Salmonella typhimurium* 878 (Rc)	MPRL
SMRE	*Salmonella minnessota* R595 (Re)	List
SMLA	*Salmonella minnessota* R595 Lipid A	List

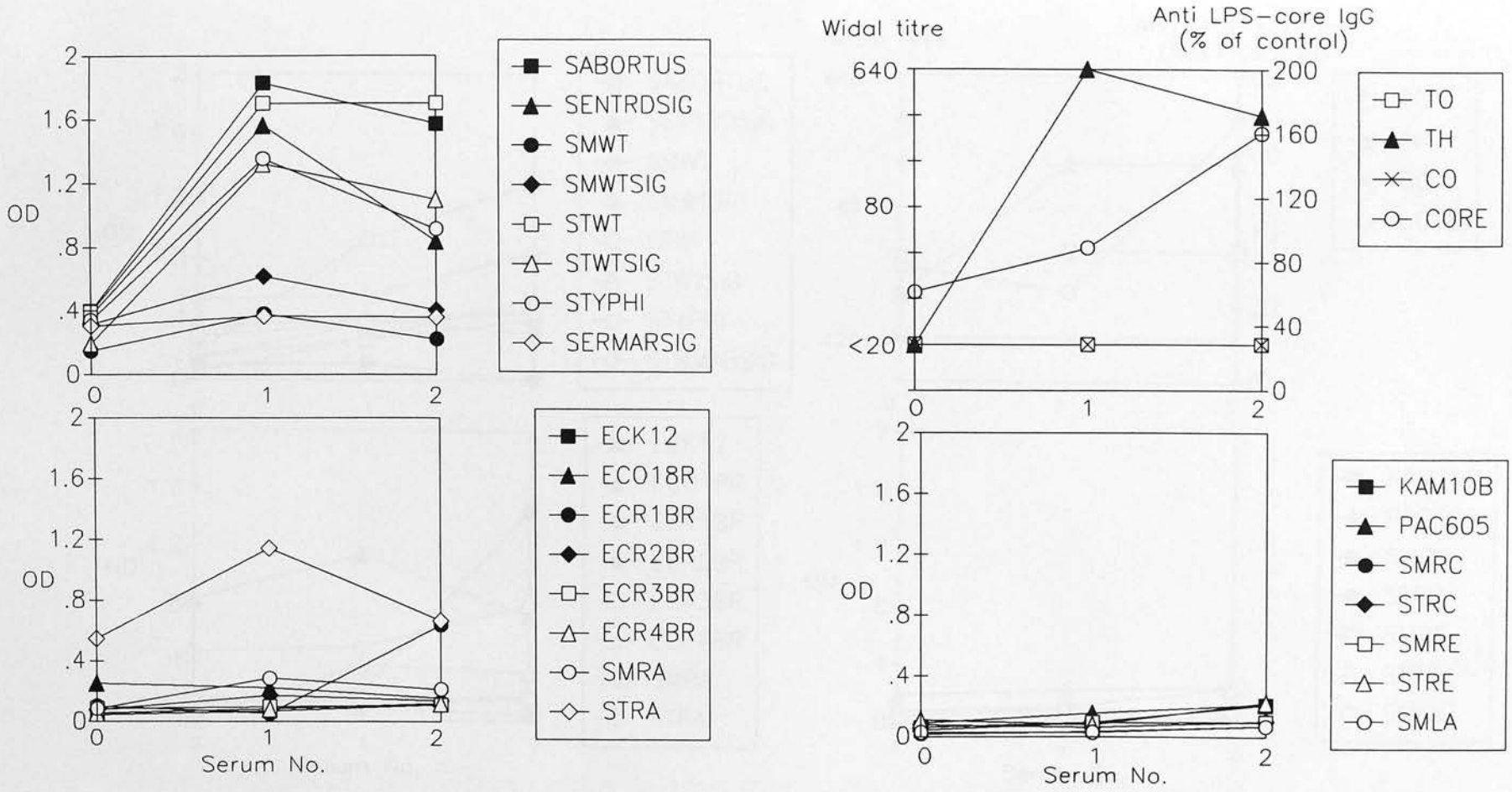


Figure 3.3 Results of Antibody tests on sera from student AB immunised with monovalent typhoid vaccine. Serum samples were taken as follows: 0 = before first dose; 1 = before second dose; 2 = 4 weeks after second dose. Antigens are as follows: TO = *S. typhi* O; TH = *S. typhi* H; CO = *S paratyphi*-C O (all Widal assays); Core = Polymyxin-LPS-core cocktail IgG ELISA (expressed as % of control); Other antigens are single chemotype polymyxin-LPS IgG ELISA (as listed in table 3.1) and results expressed as ELISA OD.

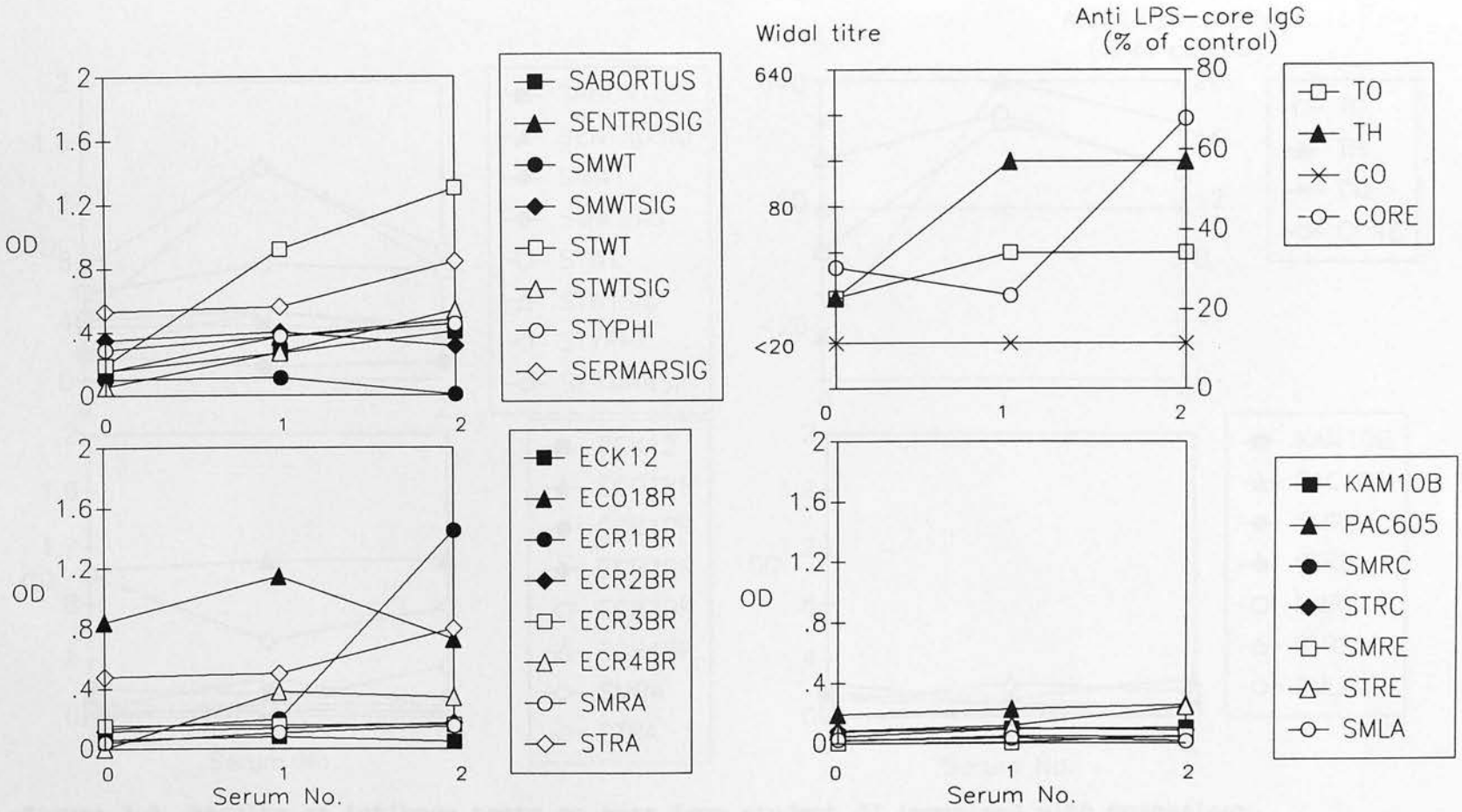


Figure 3.4 Results of Antibody tests on sera from soldier YG immunised with typhoid/paratyphoid (TAB) vaccine and tetanus toxoid. Serum samples were taken as follows: 0 = 19 days after first dose; 1 = 1 day after second dose; 2 = 10 weeks after second dose. Antigens are as follows: TO = *S. typhi* O; TH = *S. typhi* H; CO = *S paratyphi*-C O (all Widal assays); Core = Polymyxin-LPS-core cocktail IgG ELISA (expressed as % of control); Other antigens are single chemotype polymyxin-LPS IgG ELISA (as listed in table 3.1) and results expressed as ELISA OD.

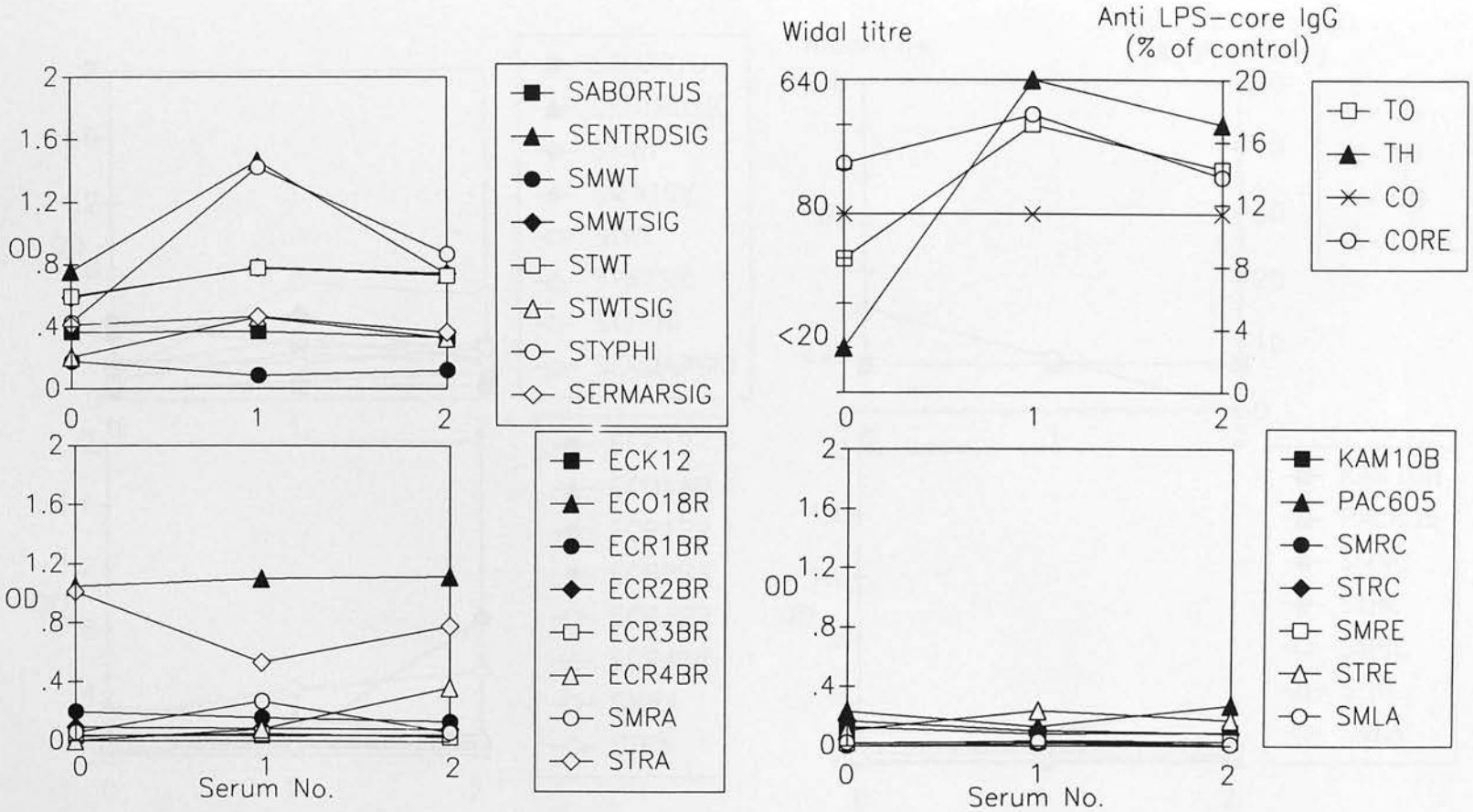


Figure 3.5 Results of Antibody tests on sera from student JJ immunised with monovalent typhoid vaccine. Serum samples were taken as follows: 0 = before first dose; 1 = before second dose; 2 = 3 weeks after second dose. Antigens are as follows: TO = *S. typhi* O; TH = *S. typhi* H; CO = *S paratyphi*-C O (all Widal assays); Core = Polymyxin-LPS-core cocktail IgG ELISA (expressed as % of control); Other antigens are single chemotype polymyxin-LPS IgG ELISA (as listed in table 3.1) and results expressed as ELISA OD.

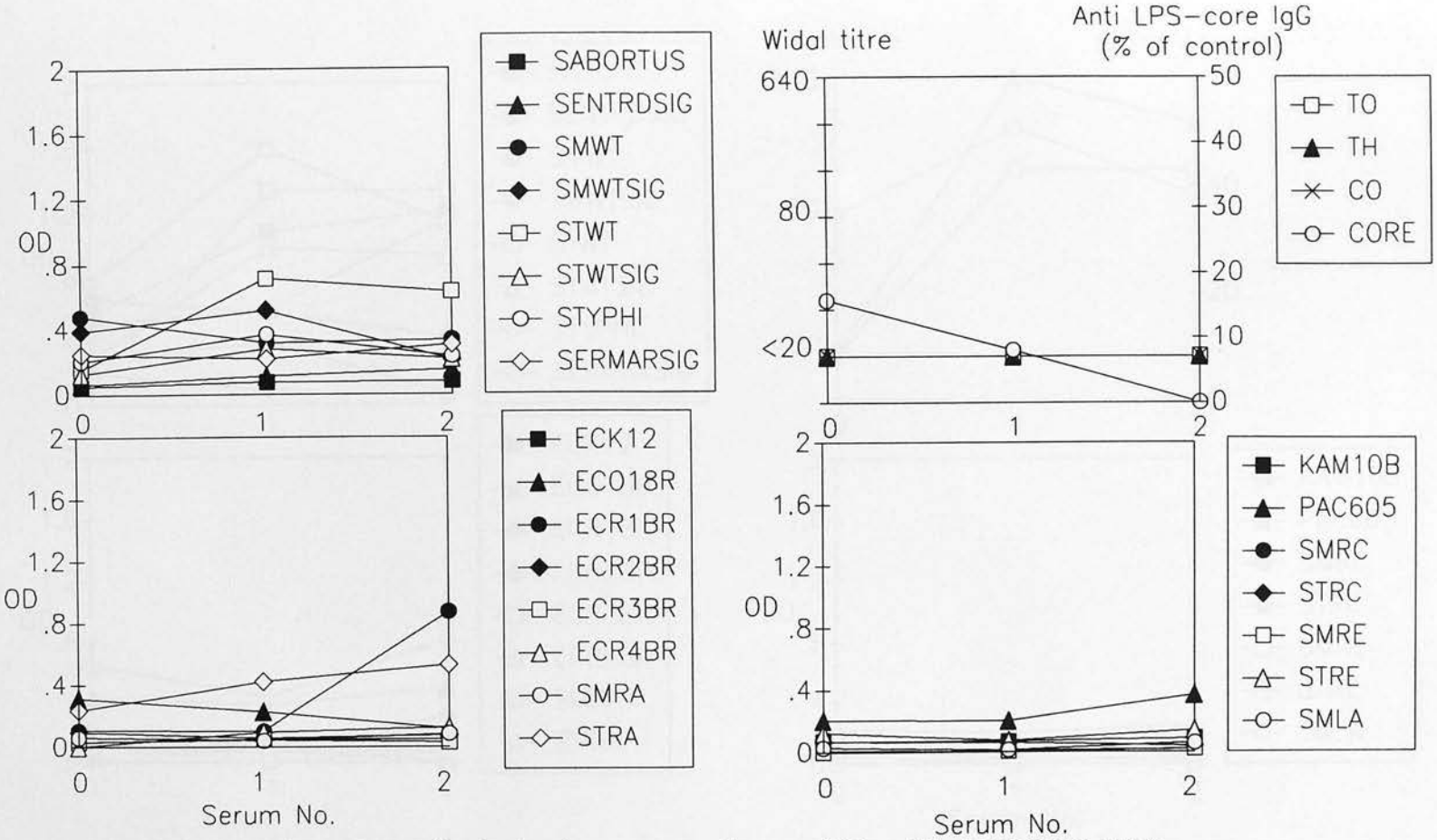


Figure 3.6 Results of Antibody tests on sera from soldier US immunised with typhoid/paratyphoid (TAB) vaccine and tetanus toxoid. Serum samples were taken as follows: 0 = 19 days after first dose; 1 = 1 day after second dose; 2 = 10 weeks after second dose. Antigens are as follows: TO = *S. typhi* O; TH = *S. typhi* H; CO = *S paratyphi*-C O (all Widal assays); Core = Polymyxin-LPS-core cocktail IgG ELISA (expressed as % of control); Other antigens are single chemotype polymyxin-LPS IgG ELISA (as listed in table 3.1) and results expressed as ELISA OD.

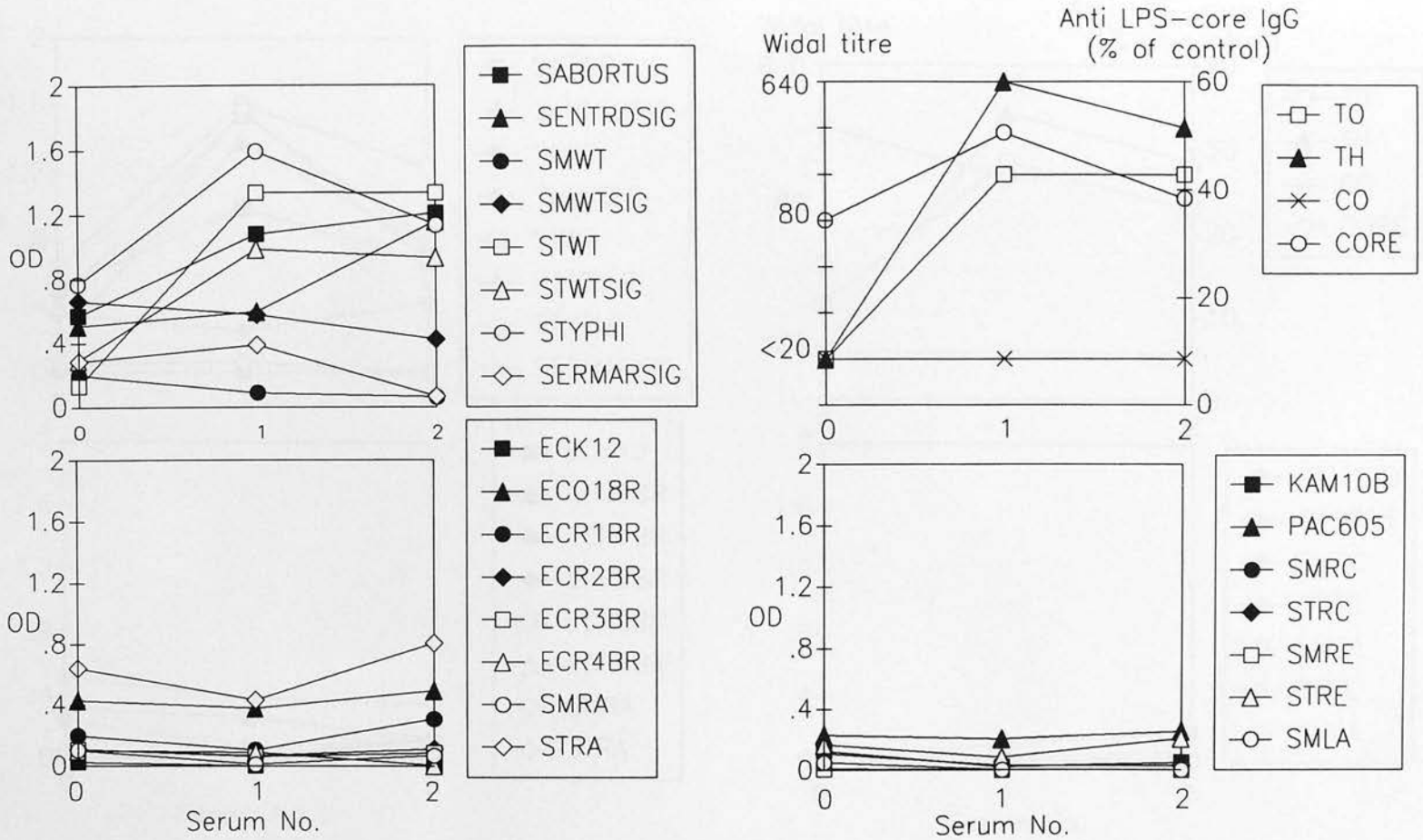


Figure 3.7 Results of Antibody tests on sera from student AT, immunised with monovalent typhoid vaccine. Serum samples were taken as follows: 0 = before first dose; 1 = before second dose; 2 = 4 weeks after second dose. Antigens are as follows: TO = *S. typhi* O; TH = *S. typhi* H; CO = *S paratyphi*-C O (all Widal assays); Core = Polymyxin-LPS-core cocktail IgG ELISA (expressed as % of control); Other antigens are single chemotype polymyxin-LPS IgG ELISA (as listed in table 3.1) and results expressed as ELISA OD.

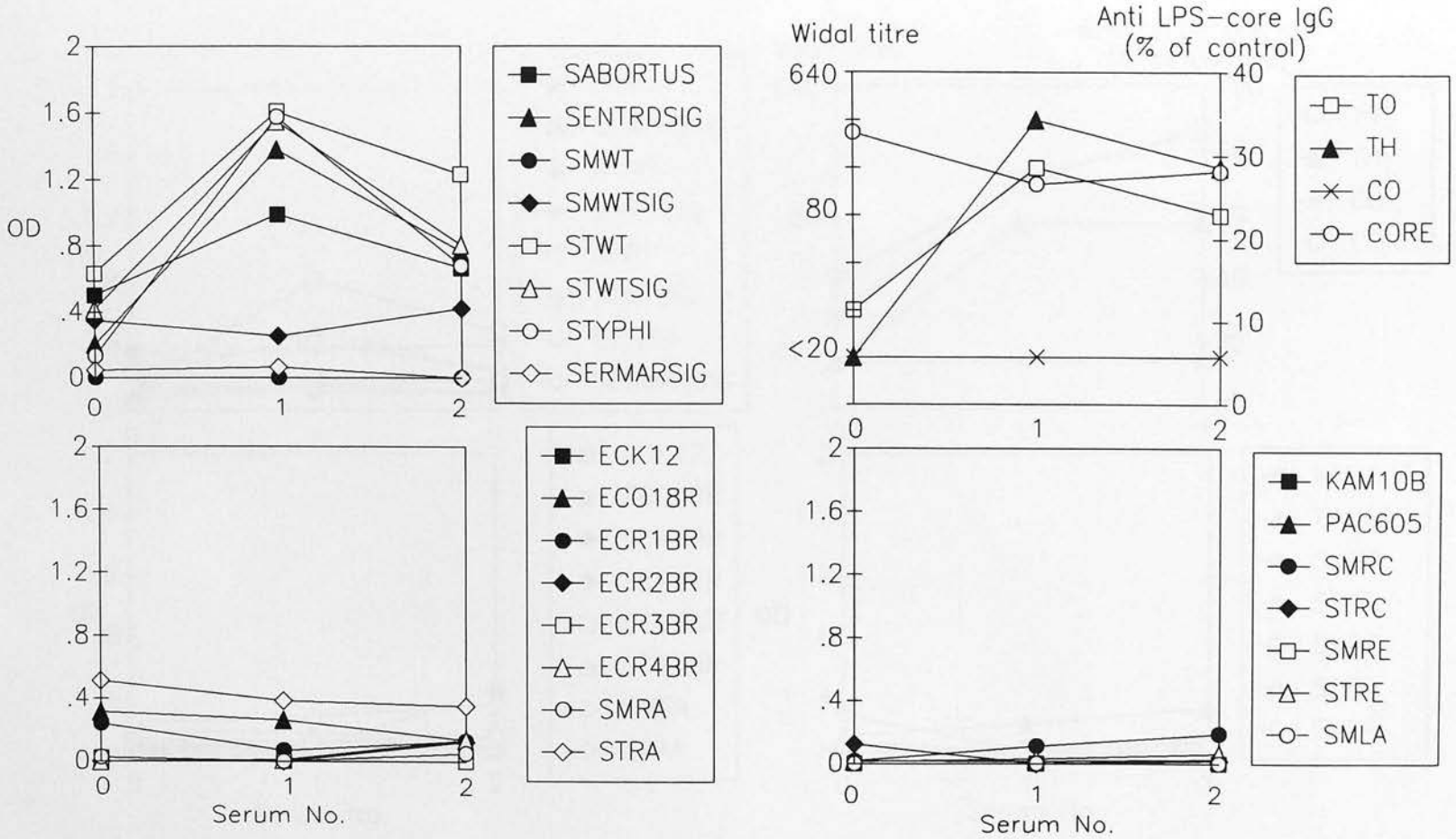


Figure 3.8 Results of Antibody tests on sera from student PU, immunised with monovalent typhoid vaccine. Serum samples were taken as follows: 0 = before first dose; 1 = before second dose; 2 = 4 weeks after second dose. Antigens are as follows: TO = *S. typhi* O; TH = *S. typhi* H; CO = *S paratyphi*-C O (all Widal assays); Core = Polymyxin-LPS-core cocktail IgG ELISA (expressed as % of control); Other antigens are single chemotype polymyxin-LPS IgG ELISA (as listed in table 3.1) and results expressed as ELISA OD.

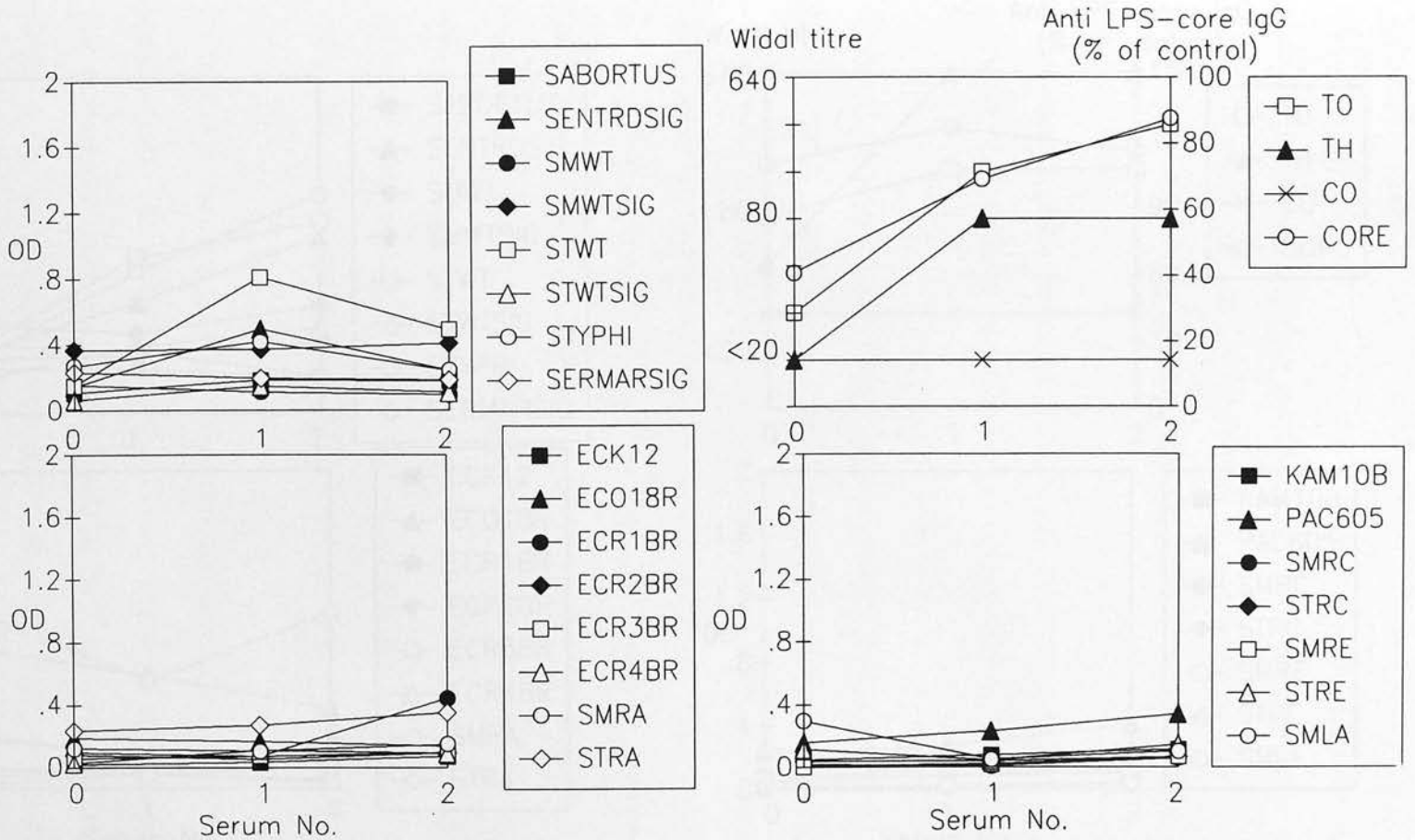


Figure 3.9 Results of Antibody tests on sera from soldier RW immunised with typhoid/paratyphoid (TAB) vaccine and tetanus toxoid. Serum samples were taken as follows: 0 = 19 days after first dose; 1 = 1 day after second dose; 2 = 10 weeks after second dose. Antigens are as follows: TO = *S. typhi* O; TH = *S. typhi* H; CO = *S paratyphi*-C O (all Widal assays); Core = Polymyxin-LPS-core cocktail IgG ELISA (expressed as % of control); Other antigens are single chemotype polymyxin-LPS IgG ELISA (as listed in table 3.1) and results expressed as ELISA OD.

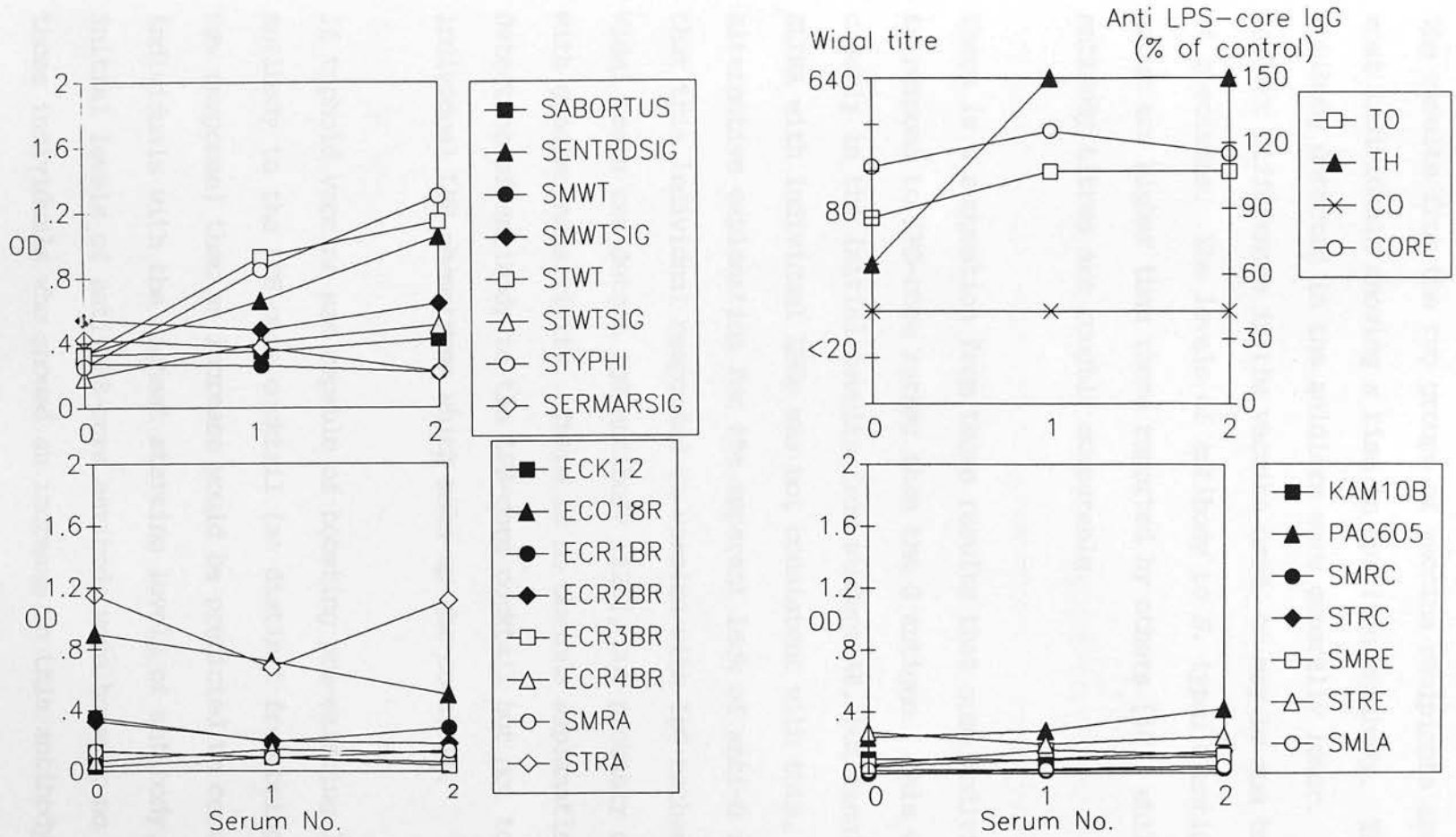


Figure 3.10 Results of Antibody tests on sera from student NW, immunised with monovalent typhoid vaccine. Serum samples were taken as follows: 0 = before first dose; 1 = before second dose; 2 = 4 weeks after second dose. Antigens are as follows: TO = *S. typhi* O; TH = *S. typhi* H; CO = *S paratyphi*-C O (all Widal assays); Core = Polymyxin-LPS-core cocktail IgG ELISA (expressed as % of control); Other antigens are single chemotype polymyxin-LPS IgG ELISA (as listed in table 3.1) and results expressed as ELISA OD.

The results from the two groups of vaccine recipients are similar, most individuals showing a rise in specific antibody. The levels of antibody detected in the soldiers were generally lower. This may reflect differences in the vaccine used, or may be due to the timing of specimens. The levels of antibody to *S. typhi* O antigen in either group are higher than those reported by others [18], while the H antibody titres are roughly comparable.

There is a suggestion from these results that some individuals tend to respond to LPS-core rather than the O antigen. This was seen most clearly in the initial results from student AB, but results of the ELISA with individual LPSs was not consistent with this. An alternative explanation for the apparent lack of anti-O response is that this individual responded to vaccine with IgG rather than IgM. Widal tests can detect IgG antibody [123], but probably detects IgM with greater sensitivity. There is no obvious explanation for the detection of antibody to the LPS-core cocktail but not to the individual LPS chemotypes which make up the cocktail.

If typhoid vaccine was capable of boosting pre-existing levels of antibody to the LPS-core cocktail (as distinct from bringing about a new response) then an increase would be predicted to occur in those individuals with the highest starting levels of antibody. The initial levels of anti-LPS-core antibody were however no higher in those individuals who showed an increase in this antibody than in those who did not (figures 3.1 - 3.2). This is in agreement with the work of Demaria et al [57] and Ziegler [264], who found that the

antibody response to *E. coli* J5 or *Salmonella minnesota* Re 595 immunisation was higher in individuals whose pre-immunisation titre was low. They used passive haemagglutination to measure the antibody response, and interpreted the lack of booster effect as indicating a T-independent and therefore primarily IgM response. Direct measurement of IgM levels to LPS-core following J5 or Re 595 or typhoid immunisation would be of interest.

The antibody response to immunisation varies considerably among the individuals tested here. Similar variation is found with other vaccines, eg with hepatitis B vaccine [43] there is variation in the response related to age and sex, with weaker responses in older individuals, and slower responses in males. Since the individuals tested here were all in their late teens or early twenties, and included an excess of males (soldiers) the results may therefore be biased. It is possible that older individuals, with a greater chance of prior exposure to Gram-negative infection, may tend to respond with less specific and more cross-reactive antibody, including perhaps anti-LPS-core antibody.

There is no overall increase in the level of antibody to LPS-core in the vaccine recipients reported here, and none of the individuals who show a rise reached the 400% of control level which was the cut-off point for selecting sera for the manufacture of anti-LPS-core IgG. There is therefore no evidence to support the idea that typhoid vaccine might be used to boost anti-LPS-core antibody levels in blood donors or in patients at risk of endotoxaemia. On the contrary, the data support the conventional view that the antibody response to a smooth LPS is dominated by antibodies to the O antigen.

CHAPTER 4

STUDIES ON IGG ANTIBODY IN URINE

It has been suggested that measuring urinary antibody to a mixed heat-killed coliform antigen preparation could be used as an aid in the diagnosis of UTI [156]. I decided to test this hypothesis, and to investigate the nature of the antibody found in urine during UTI. An initial question, relating this work to the rest of this thesis, was to what extent the antibody detected was directed against LPS-core.

Previous studies of antibody in the urine during UTI have focused mainly on specific responses to organisms isolated from individual patients [194,216,219,227], and have often had the aim of trying to distinguish between cystitis and pyelonephritis [194,219]. Other workers have measured total IgA and secretory IgA in the urine in patients with UTI [68,69]. It has been shown, at least in pyelonephritis, that the antibody found in the urine is part of a systemic immune response, and that there may be some local production of specific secretory IgA in the urinary tract [216,219]. Antibody response to antigens which are not strain specific, such as outer membrane proteins [169] lipid A [141] or pili [202] have been measured in the serum in relation to urinary infection. Serum antibodies to these non-strain-specific antigens have been found to be raised after pyelonephritis and, to a lesser extent, cystitis.

It is not clear whether antibody in the urine plays a significant part in host defence against UTI. Attachment of bacteria to the urinary tract epithelium is a key step in the pathogenesis of UTI

which could be blocked by antibody, but antibodies to the pili which are thought to act as adhesins have not been found in the urine [202]. Deficiency of urinary antibody production does not appear to be an important factor in recurrent UTI in adults [69,216], though it may be important in children [68].

The main class of antibody detected in urine with the mixed heat-killed coliform ELISA was IgG [156]. I decided therefore to concentrate just on IgG, since the mechanism of production of IgA was probably different and would complicate the interpretation of results.

Urine samples

Control samples were obtained from 16 asymptomatic students. Seventy two urine specimens were collected from students with symptoms of urinary infection attending general practitioners at the University of Edinburgh Students' Health Centre. None of these patients was thought to have pyelonephritis. One patient who was known to be taking an antibiotic at the time was excluded. Most of these patients were subsequently treated with antibiotics, and none returned because of persisting symptoms. The specimens were collected under the supervision of an experienced nurse, and were then brought directly to the clinical bacteriology laboratory in Department of Medical Microbiology by the patient.

Microscopy was performed using an inverted microscope. Ten pus cells per field (equivalent to 20 per mm^3) was scored as positive. Cultures (standard loop) containing > 10^5 cfu/ml of a single bacterial species were considered positive. Cultures containing between 10^4 and 10^5 cfu/ml would be considered doubtful in this group of patients.

Urine samples from patients with Gram-positive infection were selected from among all specimens sent to the clinical bacteriology laboratory by general practitioners.

Urine samples were stored at 4°C overnight, then centrifuged at 4000g

for 10 minutes and the supernatant stored at -20°C as three separate 1 ml volumes.

Urine samples with simultaneously taken serum or plasma samples

Thes samples were obtained from patients attending the recurrent UTI clinic at the City Hospital, Edinburgh. Many of these patients were on continuous low-dose antibiotics. Diagnostic bacteriological testing of these samples was carried out at the City Hospital bacteriology laboratory. These samples were collected as two distinct groups:

Group 1: Urine and serum samples from 44 unselected patients attending Dr JA Gray's out-patient clinic were collected in 1991. These urines were centrifuged and supernatants stored as above. Only three of these patients had microbiologically confirmed UTI.

Group 2: Urine and plasma samples from 8 patients with microbiologically confirmed UTI and 14 patients without confirmed UTI were selected from a large number of specimens collected in 1983 and 1984 by Dr CC Blackwell. In these cases the urines were not centrifuged before freezing.

All urine and serum or plasma samples were stored at -20°C.

Mixed Heat-killed Coliform ELISA plates

The preparation of these plates was based on the method described by McKenzie and Young [156]. Six isolates of coliform organisms (four *E. coli*, one *Klebsiella pneumoniae*, and one *Proteus mirabilis*) were obtained from urine samples submitted to the diagnostic laboratory of the Department of Medical Microbiology. (The organisms were deposited in the MPRL freeze-dried collection, reference numbers MPRL 2287 - 2292). These organisms were chosen to represent the range of Gram-negative bacteria isolated from urine specimens. Each organisms was grown overnight in nutrient broth at 37°C, then washed and resuspended in PBS. The concentration was adjusted so that a 1 in 10 dilution had an OD of 0.25 at 540 nm, and heated at 100°C for 30 min. The six preparations were mixed together in equal proportions. The mixture was diluted 1:10 in coating buffer, and 100µl added per well to Nunc polysorb 8-well ELISA strips. After overnight incubation at room temperature the strips were washed four times with wash buffer.

Urine ELISA assay for anti-bacterial IgG

Assays were performed using polymyxin-LPS-core plates (see general materials and methods) or mixed heat-killed coliform plates as described above. Duplicate aliquots of urine (100µl) were dispensed into coated wells and incubated for 1 hour at 37°C. Plates were then washed four times with wash buffer, 100µl of alkaline phosphatase conjugated goat anti-human IgG (ICN, diluted 1 in 500 in ELISA diluent with 0.5% bovine serum albumin) added, incubated for a further hour at 37°C, and again washed four times. P-nitrophenyl

phosphate (Sigma, 100 µl of a 1mg/ml solution in 0.05M sodium carbonate, 1mM magnesium chloride, pH 9.8) was added and the OD read after 30 minutes at room temperature. Each assay included duplicate blank wells with no urine added. Wells without antigen but washed as above were used as negative controls.

Absorption of antibody with LPS-core cocktail plates

Samples (100µl) of antibody-positive urine were added in duplicate to wells coated with polymyxin-LPS-core cocktail antigen and incubated at 37°C for one hour. Incubation and transfer were repeated twice so that each sample had been incubated for a total of 3 hours in three separate wells. Antibody was then measured by ELISA using plates coated with the mixed heat-killed coliform antigen preparation. Antibody results were compared with those for unabsorbed urine and for urine that had been absorbed in control wells (wells containing no polymyxin-LPS-core cocktail but washed and post-coated in the same way as coated wells).

Urinary total IgG assay

Nunc Maxisorb plates were coated with affinity purified goat anti-human-IgG (Zymed) (diluted 1 in 2000 in 0.05M carbonate/bicarbonate buffer, pH 9.6) by adding 100µl to each well and incubating overnight at room temperature. Each well was then washed four times with wash buffer. Wells were then post-coated for 2 h at room temperature with 100µl per well of 5% bovine serum albumin in PBS. Plates were then washed four times in wash buffer and stored at -20°C. Urine samples

were diluted 1 in 3 in PBS and duplicate 100µl samples added to wells in the coated plate. Human serum protein calibrator (Dakopatts) was diluted in PBS to give concentrations of 100, 25, 6.3, 1.6, 0.4, 0.1, and 0.025 µg/ml, and 100 µl samples added in triplicate to each assay plate. Coated wells to which PBS alone was added were included as blanks. Plates were incubated for 1 hour at 37°C, and then washed four times. Alkaline phosphatase labelled goat anti-human IgG (ICN) was diluted 1 in 500 in ELISA diluent with 0.5% bovine serum albumin and 100µl added to each well, incubated for 1 h at 37°C and washed four times. P-nitrophenyl phosphate (Sigma, 100 µl of a 1mg/ml solution in 0.05M sodium carbonate, 1mM magnesium chloride, pH 9.8) was added and the OD measured, after 120 minutes at room temperature, on an Anthos plate reader. IgG concentrations were calculated from the standard curve by the plate reader's on-board software, taking into account the dilution of the urine.

Calculation of predictive value of antibody testS

This calculation was performed in the same way as other comparable studies [112,135,157]:

Sensitivity = true positives /(true positives + false negatives)

Specificity = true negatives /(true negatives + false positives)

Positive predictive value = true positives /(true positives + false positives)

Negative predictive value = true negatives /(true negatives + false negatives)

IgG antibodies to mixed-heat-killed coliform antigen and polymyxin-LPS-core cocktail antigen in urine.

For all but one of the asymptomatic controls both assays gave OD values of < 0.1. One male asymptomatic control did have consistently high titres of urinary antibody to both antigens, with OD values of > 1.0. This individual did not have covert urinary tract infection, haematuria, proteinuria, hypertension, or any other evidence of renal or other disease.

Of the 72 samples from symptomatic patients, 19 were culture positive, and the remaining 53 were culture negative. All the positive cultures were of coliform organisms, and in all cases they were present at > 10^5 cfu/ml. Pus cells were seen in 17 of the 19 culture-positive specimens, and in one of the 53 culture-negative specimens.

IgG antibody to both the LPS-core cocktail antigen and the mixed heat-killed coliform antigen was detected in most of the symptomatic patients (figure 4.1). Significantly more antibody was found in specimens which were culture positive than in those which were culture negative. For the LPS-core antigen, $t = 3.65$; $p < 0.001$, while for the mixed heat-killed coliform antigen $t = 3.55$; $p < 0.001$. The usefulness of antibody as a predictor of positive culture was, however, low. For the LPS-core antigen, an OD of > 0.6 had a sensitivity (defined as the proportion of the culture-positive

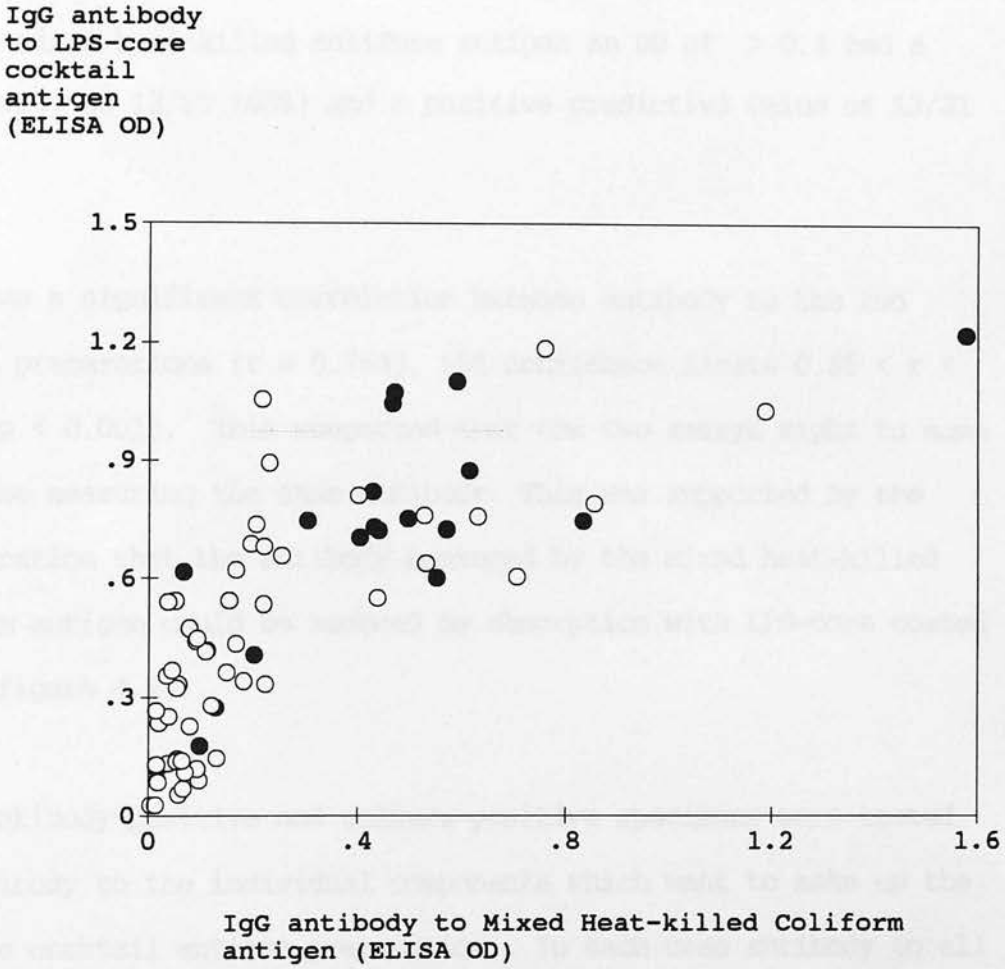


Figure 4.1 Correlation between IgG antibodies (ELISA OD) to mixed heat-killed coliform antigen and LPS-core cocktail antigen in 72 urine samples from symptomatic patients.
● = culture positive; ○ = culture negative.

samples which were picked out by the ELISA test) of 15/19 (79%) and a positive predictive value (defined as the number of samples picked out by the ELISA test which were culture positive) of 15/30 (50%). For the mixed heat-killed coliform antigen an OD of > 0.4 had a sensitivity of 13/19 (68%) and a positive predictive value of 13/21 (62%).

There was a significant correlation between antibody to the two antigen preparations ($r = 0.7633$, 95% confidence limits $0.65 < r < 0.85$, $p < 0.001$). This suggested that the two assays might to some extent be measuring the same antibody. This was supported by the demonstration that the antibody measured by the mixed heat-killed coliform antigen could be reduced by absorption with LPS-core coated wells (figure 4.2).

Eight antibody-positive and culture-positive specimens were tested for antibody to the individual components which went to make up the LPS-core cocktail antigen preparation. In each case antibody to all of the component parts of the antigen preparation could be detected. No one LPS preparation stood out as a particularly good antigen (table 4.1).

Urine samples from which > 10^5 cfu/ml of a Gram-positive organism was isolated were selected (from those submitted to the clinical bacteriology laboratory by general practitioners) and tested for antibody to LPS-core and mixed heat-killed coliform antigens. These antibody levels in these samples (table 4.2) were similar to those found in urines with Gram-negative bacterial infection. The mean OD

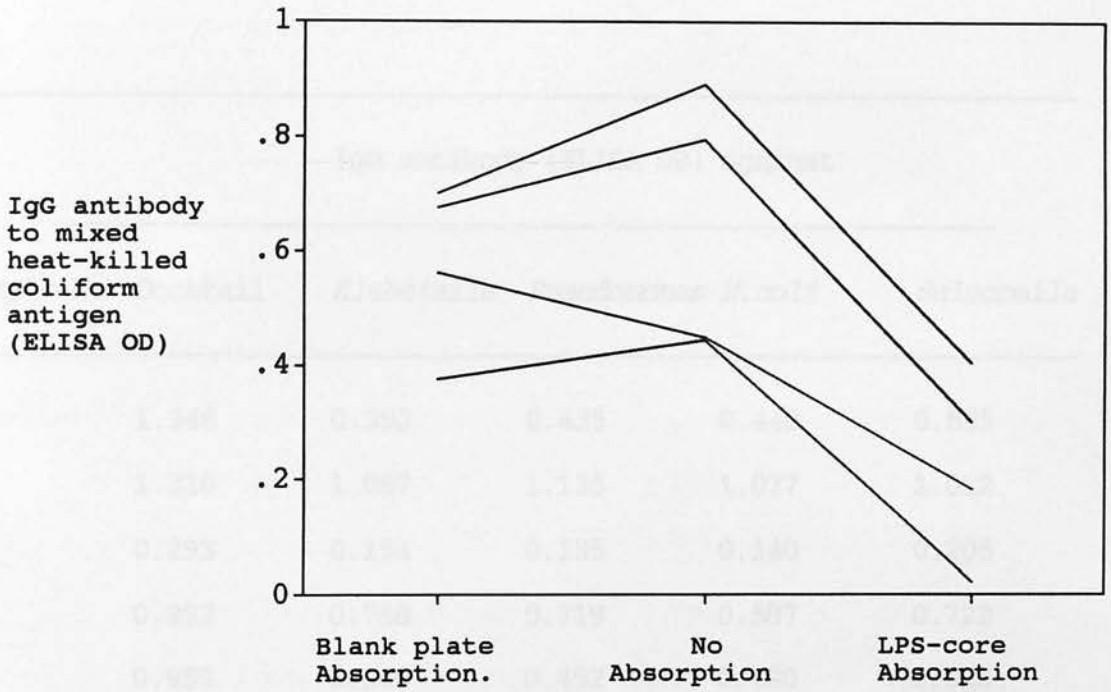


Figure 4.2 Reduction in IgG antibody (ELISA OD) to mixed heat-killed coliform antigen in four urine samples following absorption for three one-hour periods in wells coated with LPS-core cocktail antigen or blank plates.

Table 4.1 IgG antibody (ELISA OD) to polymyxin-LPS-core cocktail and individual polymyxin complexed rough LPS preparations in eight culture-positive urine samples.

	IgG antibody (ELISA OD) against				
Sample No	Cocktail	*Klebsiella*	*Pseudomonas*	*E.coli*	*Salmonella*
1	1.346	0.393	0.435	0.440	0.535
2	1.310	1.087	1.135	1.077	1.012
3	0.293	0.154	0.135	0.140	0.205
4	0.992	0.758	0.719	0.587	0.722
5	0.969	0.340	0.452	0.400	0.528
6	0.888	0.507	0.480	0.513	0.473
7	0.995	0.463	0.478	0.514	0.672
8	1.302	0.358	0.256	0.300	0.676

Table 4.2 IgG antibody (ELISA OD) to mixed heat-killed coliform antigen and polymyxin-LPS-core cocktail antigen in six urine samples from which $>10^5$ cfu/ml of a Gram-positive organism was isolated.

		IgG antibody (ELISA OD) detected with:	
Sample	Organism isolated	Mixed heat-killed coliform	LPS-core cocktail
1	*Enterococcus* sp	0.256	0.571
2	*Enterococcus* sp.	0.589	1.054
3	*Staphylococcus saprophyticus*	0.265	0.584
4	*Staphylococcus saprophyticus*	0.1	0.227
5	*Staphylococcus saprophyticus*	0.688	1.235
6	Group G Streptococcus	0.528	1.091

against the mixed heat-killed coliform antigen was 0.404 for the Gram-positive urines, and 0.458 for the Gram-negative urines. With the LPS-core cocktail antigen the mean OD for the Gram-positives was 0.794 while for the Gram-negatives it was 0.708. The finding of antibody to Gram-negative bacterial antigens in Gram-positive infection implied that the antibody detected was not due to a specific immune response to the infecting organism.

Total IgG levels in urine samples

One possible explanation for the presence of antibody in urine is that it was simply the result of non-specific leakage of serum proteins into the urine as a result of inflammation. This would explain the finding of antibody to Gram-negative bacteria in Gram-positive infection. A capture assay for total IgG in urine was therefore developed, and applied to some of the urine samples. An example of a standard curve for this assay is shown in figure 4.3.

Sixty-three of the original 72 urines from symptomatic patients were still available as frozen aliquots which had not been thawed. Eighteen of these were culture-positive specimens. IgG concentration (expressed as a logarithm) had a statistically significant correlation with IgG antibody to mixed heat-killed coliform antigen ($r = 0.52$, 95% confidence interval 0.32 - 0.68, $p < 0.001$) and with IgG antibody to LPS-core cocktail antigen ($r = 0.68$, 95% confidence limits 0.52 - 0.79, $p < 0.001$) (figure 4.4). Total IgG tended to be higher in culture positive specimens (Kruskal-Wallis $H = 17.4$, $p = 0.00003$). In this group of specimens an IgG of > 1

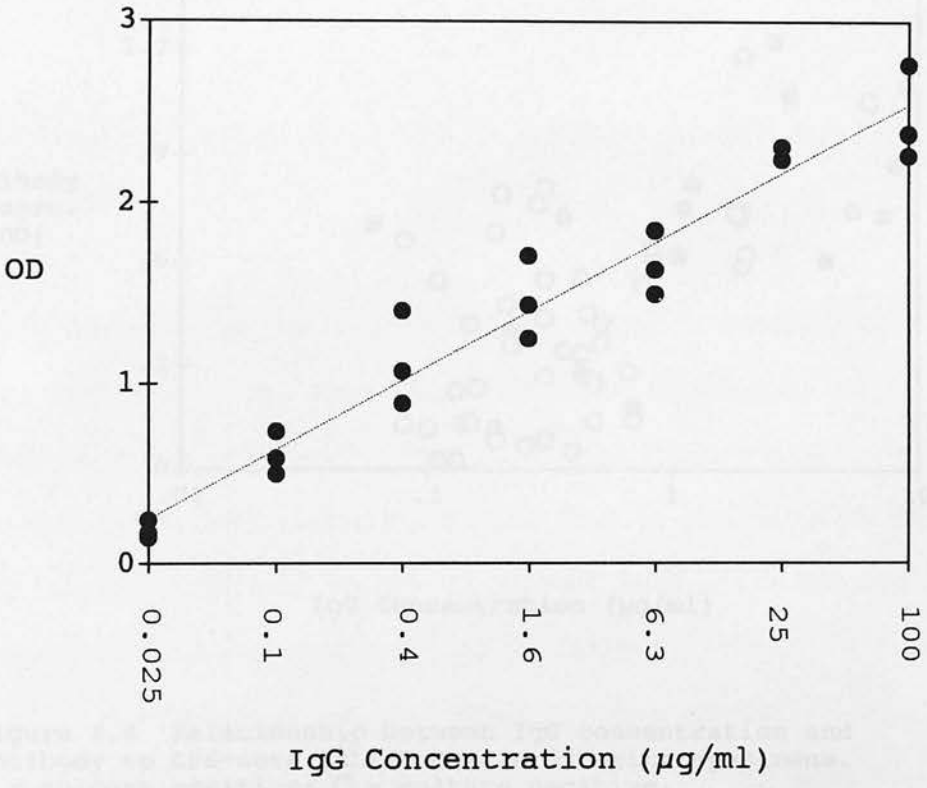


Figure 4.3 Example of standard curve for urinary IgG assay showing log-linear regression line.

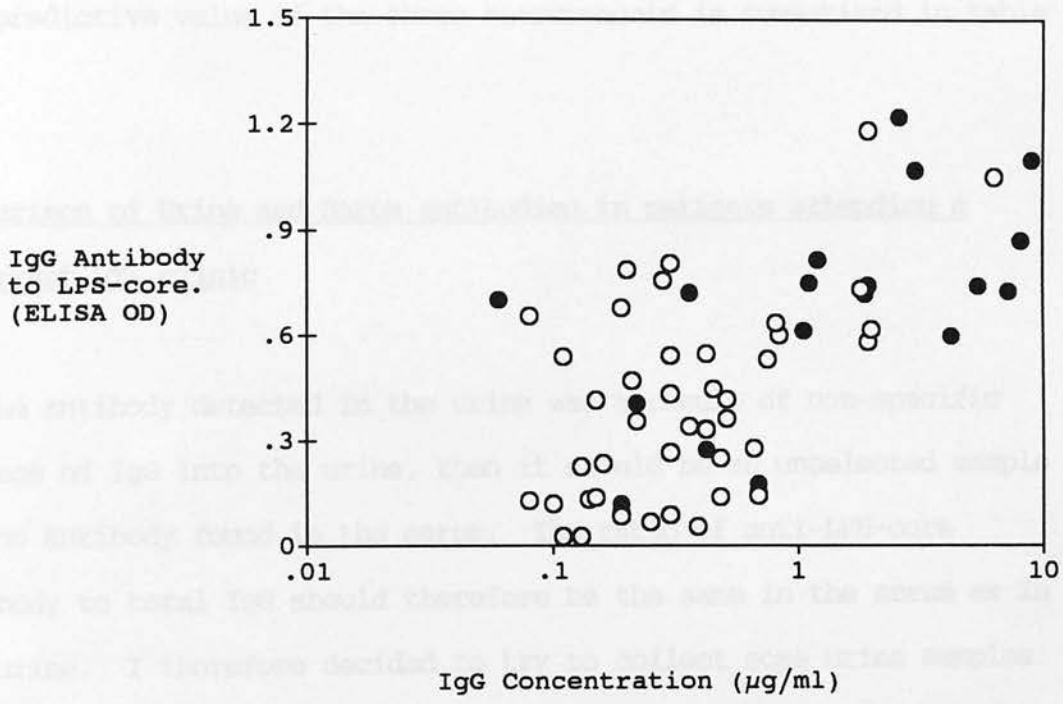


Figure 4.4 Relationship between IgG concentration and antibody to LPS-core (ELISA OD) in 63 urine specimens. ● = culture positive; O = culture negative.

µg/ml had a sensitivity for positive culture of 11/18 (61%) and a positive predictive value of 11/17 (65%), implying that it is roughly as good (as a predictor of positive culture) as measurements of antibody to either LPS-core or mixed heat-killed coliform antigens. The predictive value of the three measurements is summarised in table 4.3.

Comparison of Urine and Serum antibodies in patients attending a recurrent UTI clinic

If the antibody detected in the urine was a result of non-specific leakage of IgG into the urine, then it should be an unselected sample of the antibody found in the serum. The ratio of anti-LPS-core antibody to total IgG should therefore be the same in the serum as in the urine. I therefore decided to try to collect some urine samples with simultaneously taken serum samples to investigate this possibility. Unfortunately the general practitioners at the University Health Centre were unwilling to subject their patients to venepuncture for this purpose. I therefore arranged to collect specimens from 44 patients attending Dr JA Gray's recurrent UTI clinic at the City Hospital Edinburgh. Only 3 of the 44 patients (group 1) had positive culture, and therefore some older samples and controls (group 2) were selected from a collection obtained from the same source by Dr CC Blackwell.

The urine samples were first tested for anti-LPS-core and total IgG as before. The results are shown in figures 4.5 and 4.6. As in the samples from students with urinary symptoms, there was a correlation

Table 4.3 Comparisons of tests for the prediction of positive culture in 72 urine samples from students with symptoms of UTI.

Test	Sensitivity	Specificity	Predictive value for positive culture	
			Positive	Negative
mixed heat-killed coliform ELISA OD > 0.4	68%	85%	62%	90%
polymixyn-LPS-core cocktail ELISA OD > 0.6	79%	72%	50%	93%
IgG[a] >1 µg/ml	61%	87%	65%	85%
Pus cells > 20/mm^3	89%	98%	94%	96%

[a]Only 63 samples tested.

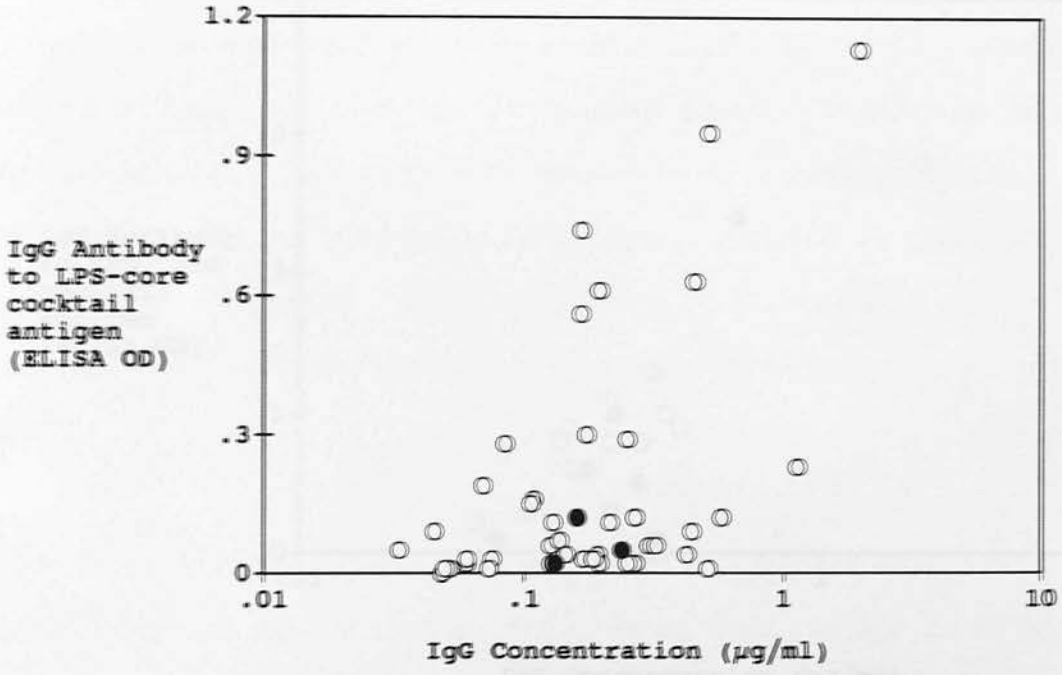


Figure 4.5 Relationship between IgG concentration and antibody to LPS-core cocktail antigen (ELISA OD) in urine specimens from recurrent UTI patients group 1 (n=44)
● = culture positive; ○ = culture negative.

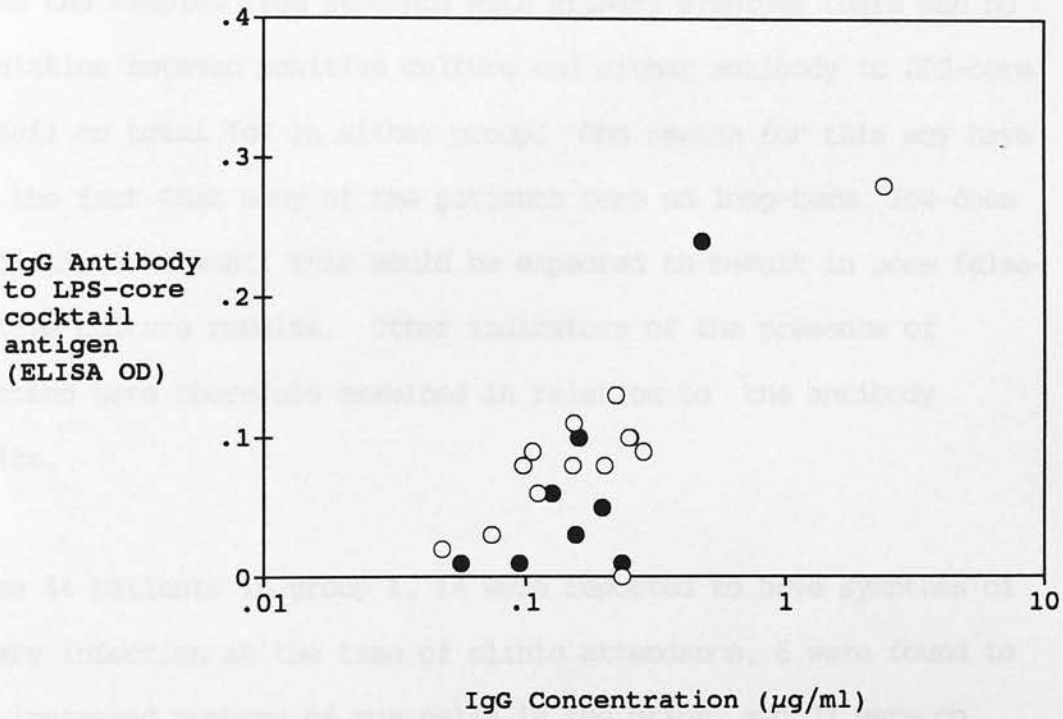


Figure 4.6 Relationship between IgG concentration and antibody to LPS-core cocktail antigen (ELISA OD) in urine specimens from recurrent UTI patients group 2 (n=22). ● = culture positive; ○ = culture negative.

between antibody to LPS-core cocktail and total IgG in these samples. In the 44 urines which I collected (group 1), $r = 0.58$, $P < 0.001$, 95% confidence interval 0.34 - 0.74. In the 22 urines obtained from Dr Blackwell (group 2) $r = 0.75$, $P < 0.001$, 95% confidence interval 0.48 - 0.89.

Unlike the samples from students with urinary symptoms there was no association between positive culture and either antibody to LPS-core cocktail or total IgG in either group. One reason for this may have been the fact that many of the patients were on long-term, low-dose antibiotic treatment. This would be expected to result in some false-negative culture results. Other indicators of the presence of infection were therefore examined in relation to the antibody results.

Of the 44 patients in group 1, 14 were reported to have symptoms of urinary infection at the time of clinic attendance, 6 were found to have increased numbers of pus cells in the urine, and 27 were on antibiotics. Results of analysis of the IgG anti-LPS-core and total IgG assays on the urine for differences related to these factors are shown in table 4.4. The only statistically significant difference observed was that patients on long term antibiotics tended to have higher levels of urinary IgG.

Serum IgG anti-LPS-core antibody was measured in 42 of the 44 sera from group 1. The antibody level was raised with a median value of 247.95 compared to a median of 100 for the blood donor population. There was a tendency for the patients on long-term antibiotic

Table 4.4 Analysis of the results of urine antibody assays by clinical indices on group 1 of the recurrent UTI patients (n=44). *P* values derived from Wilcoxon two-sample test.

	Anti LPS-core IgG		Total IgG	
Index	Mean OD	*P*	Mean OD	*P*
Pus cells in urine:				
Increased	0.113	0.97	0.343	0.52
No increase	0.182		0.246	
Symptoms of UTI:				
Present	0.258	0.36	0.389	0.34
Absent	0.133		0.198	
Long term antibiotics:				
In use	0.219	0.09	0.329	0.04
Not in use	0.099		0.148	

treatment to have higher levels of this antibody (median level 378) than those who were not on antibiotics (median level 256), but this difference did not reach statistical significance (Wilcoxon two-sample test gives $P = 0.34$).

If anti-LPS-core antibody in the urine was simply a result of non-specific leakage of antibody into the urine it follows that the ratio of anti-LPS-core to total IgG antibody should be the same in both serum and urine. It was not however clear how the ratios should be calculated since the anti-LPS-core measurements were not on linear scales. The urinary anti-LPS-core IgG measurements were simply ELISA OD values, which probably bore a logarithmic relationship to the actual amount of antibody present. In addition to calculating simple ratios of the available data the results of some data transformations was therefore examined. The expected correlation between the urine and serum values of the ratio of anti-LPS-core IgG to total IgG could not however be demonstrated with either the basic data nor with any transformation. Figure 4.7 shows the result obtained with one data transformation, where an attempt was made to make the urine anti-LPS-core IgG data linear by expressing the results as the exponent of the OD value, $e^{(OD)}$. Other transformations examined consisted of using the log of the total IgG values and log of the serum anti-LPS-core IgG values.

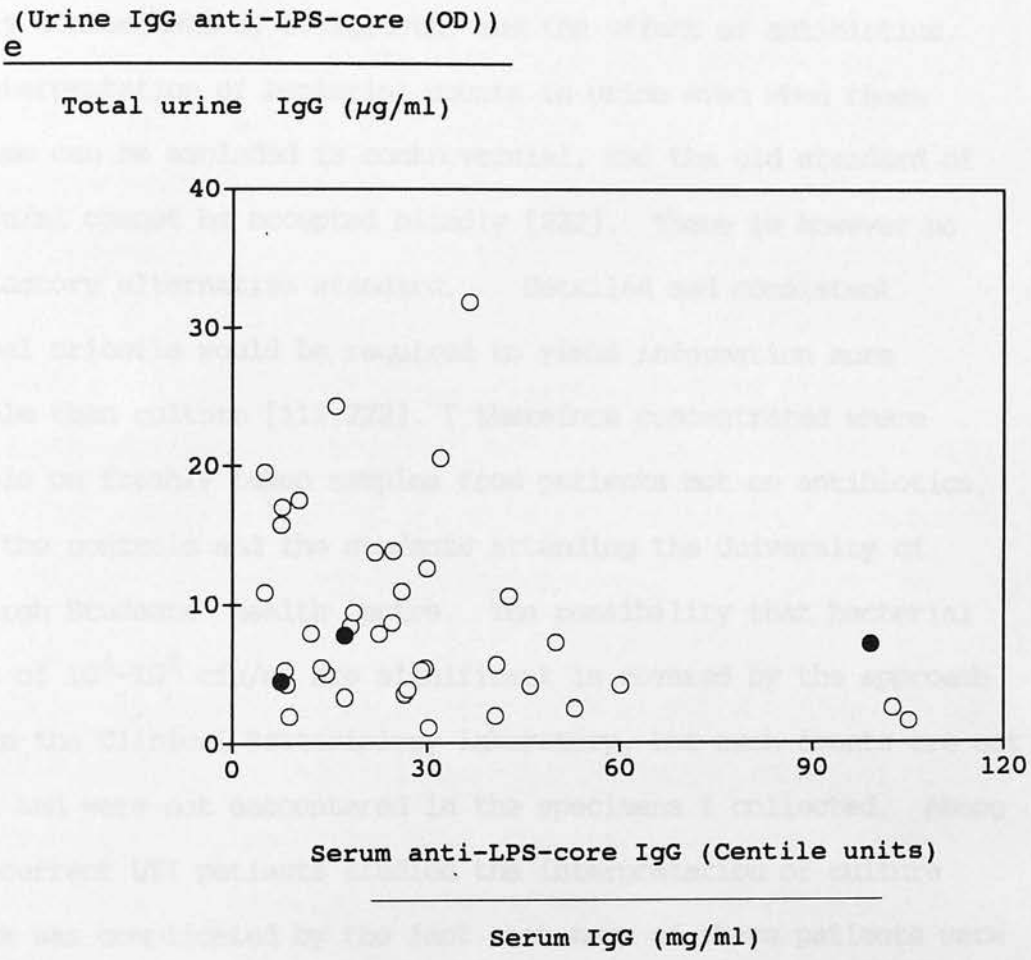


Figure 4.7 Relationship between ratio of anti-LPS-core IgG to total IgG in serum and urine of 40 patients attending a recurrent UTI clinic.
● = culture positive; ○ = culture negative.

DISCUSSION

The "gold standard" used for the diagnosis of urinary tract infection is quantitative culture. Quantitative culture of bacteria in urine can be influenced by many factors other than the presence of UTI, notably contamination, overgrowth, and the effect of antibiotics. The interpretation of bacterial counts in urine even when these problems can be excluded is controversial, and the old standard of 10^5 cfu/ml cannot be accepted blindly [222]. There is however no satisfactory alternative standard. Detailed and consistent clinical criteria would be required to yield information more reliable than culture [112,222]. I therefore concentrated where possible on freshly taken samples from patients not on antibiotics, as in the controls and the students attending the University of Edinburgh Students' Health Centre. The possibility that bacterial counts of 10^4–10^5 cfu/ml are significant is covered by the approach used in the Clinical Bacteriology laboratory, but such counts are not common and were not encountered in the specimens I collected. Among the recurrent UTI patients studied the interpretation of culture results was complicated by the fact that many of these patients were on antibiotics.

Urinary antibody is clearly associated with UTI in the symptomatic student group, but the predictive values for positive culture of the urinary antibody tests reported here are too low to be useful in diagnostic laboratories. Very similar conclusions have been reached by other workers [112,135,157].

One reason for the poor predictive value of urinary antibody measurements is the raised antibody levels found in many culture-negative specimens from symptomatic patients. Similar results were obtained by McKenzie and Young [156]. Antibacterial substances in urine could account for some false-negative cultures, but previous studies in the Clinical Bacteriology laboratory have found such substances in less than 1% of urines from the Students' Health Centre. Urethritis, or urinary tract infection with $< 10^4$ cfu/ml might be present in some case, but the absence of large numbers of pus cells in all but one of the culture negative specimens is against this. There is no satisfactory explanation for acute urinary symptoms in many patients however [223], and the finding of raised levels of antibody in this group may be an indication of some as yet undefined physical basis for the symptoms.

Antibody to LPS-core cocktail or mixed heat-killed coliform correlated with one another, and both had a similar association with positive culture in the symptomatic students. The assays could be measuring two quite separate antibodies which happen to occur together, or they could be measuring the same antibody. The demonstration that the coliform antibody can be reduced by absorption with LPS-core antigen suggests that antibody to LPS-core makes up a sizable proportion of the antibody detected by the coliform antigen. This may in part reflect the fact that other antigens in the coliform antigen will have been denatured by heat (in the case of proteins) or will be highly strain specific (in the case of O antigens). It might also indicate that antibody to LPS-core is an important part of the host immune response to UTI.

The assay used to measure urine IgG levels was fairly crude. An examination of the scatter of triplicates on the standard curve (figure 4.4) indicates an overlap between OD values obtained with some pairs of neighbouring standards. The cut-off value chosen to distinguish between culture-positive and culture-negative urines from the symptomatic patients is 1 µg/ml. Connel et al [49], using a similar assay, report 1 µg/ml as the median concentration of IgG found in urine samples from a group of genitourinary medicine patients and blood donors being tested for antibody to HIV. Lentner [1] quotes a median daily excretion of 1.93 mg of IgG in adults, equivalent to 1.6µg/ml for a mean daily urine volume of 1.2 l. The absolute values of urinary IgG reported here are therefore generally lower than those expected in asymptomatic controls. It is possible that the IgG concentration in patients with urinary infection would be low because of urine dilution. It is perhaps more likely that the crude assay employed is giving results which are too low. It would have been useful to measure urinary IgG in some asymptomatic controls, but this was not considered until the materials for the IgG assay had been exhausted. The assay might also have been improved by using urine spiked with purified IgG as the standard

Despite the limitations of the assay, the total urinary IgG did correlate with the other antibody measurements. The simplest explanation for this would be that antibody was leaking into the urinary tract in association with inflammation. The antibody to LPS-core and coliform antigens would then simply reflect the prevalence of these antibodies in the general population. Antibody to LPS-core

is known to be common in blood donors [9].

The view that the antibody detected in urine in this and similar studies [112,135,156,157] is not part of a specific immune response is supported by the fact that it is present very early in the course of infection. It is further supported by the finding of antibody to Gram-negative bacteria in Gram-positive infection. It is possible that there is a specific immune response which results in the presence of IgG in the urinary tract, but this would probably be masked by non-specific leakage of antibody.

If the antibody present is due to non-specific leakage it follows that there might well give false-positive results in patients with gonococcal or chlamydial urethritis. It is important that these infections are differentiated accurately from UTI, since they are managed quite differently. In response to this suggestion Thakker et al [231] reported negative findings with the "Uristat" test in patients with urethritis. This result may be due to the relatively small area of epithelium involved in urethritis, resulting in a much lower concentration of IgG leakage into the urine. Further studies of antibody in the urine in urethritis are required to clarify this point.

In the recurrent UTI patients antibody was not associated with positive culture. This underlines the differences between the two groups of patients. Most of the recurrent UTI patients were asymptomatic, and symptoms, when present, were probably less severe in the recurrent UTI patients than in the students with acute urinary

symptoms. Many of the recurrent UTI patients were on long-term antibiotics, which may have resulted in some false-negative cultures. The association between long-term antibiotic treatment and raised levels of urinary IgG might be because patients on long-term antibiotics were those with the more severe problems of recurrent infection, associated with chronic inflammation in the urinary tract. The finding of raised levels of serum anti-LPS-core IgG in this same subgroup also suggests that these may be the patients who have had the larger number of Gram-negative infections.

If the IgG antibody detected in urine is simply a result of non-specific leakage of serum components then the ratio of specific to total IgG should remain constant. It may appear unfortunate that the recurrent UTI patients were chosen for the comparison of serum and urine antibody levels, since the association of antibody with positive culture was not found in this group, but the hypothesis is still valid in this group as in symptomatic patients. The fact that the expected result was not obtained may be because of inaccuracies in the assays used, but may indicate that there is an alternative explanation for at least some of the IgG present in the urine. IgG may for example be produced locally in the urinary tract in some patients.

Possible clues as to the alternative ways in which IgG might get into the urine were sought by considering the clinical background to the patients who had the greatest differences between the urine and serum ratio of specific to total IgG, but no associations were apparent. If this question were to be investigated further then a more accurate

assay of urinary IgG would be required, and it would also be useful to consider a range of different specific antibodies.

The initial purpose of measuring urinary antibody to a mixture of common urinary pathogens was that it would permit rapid detection of UTI and it would be less susceptible to the problems of contamination and overgrowth than conventional bacteriology. In our hands IgG antibodies did not predict positive culture in fresh, carefully taken samples to a useful extent. Such antibody tests are therefore not likely to be useful in examining contaminated specimens or specimens where the patient has been on antibiotics. Other workers have come to similar conclusions using similar antigen preparations but measuring all antibody classes. It is possible that measuring urinary IgA or secretory IgA antibody to some shared bacterial antigen or combination of antigens would give some diagnostically useful information, but considerable further work is required to establish this. Measurement of IgG in urine might be a useful index of inflammation in the urinary tract.

CHAPTER 5

INITIAL STUDIES ON THE INTERACTION OF MABS AND HUMAN IGG PREPARATIONS WITH BLOOD-CULTURE ISOLATES OF GRAM-NEGATIVE BACILLI AND WITH ROUGH MUTANTS OF *E. COLI*

INTRODUCTION

When I first became involved with this work the production of MAbs to LPS had been under way for over 2 years, both in Edinburgh and in Basel. I set out to test the reactivity of some promising MAbs with LPS from a range of bacterial species selected from local blood culture isolates. As indicated in the general introduction, blood culture isolates may be the best available indication of the sort of LPS likely to be involved in sepsis syndrome patients, and therefore it was hoped that some of the MAbs might be widely cross-reactive with these strains. This work was then extended to examine the binding of selected MAbs to the known core types of *E. coli*, and to test some alternative methods of detecting the binding of antibody to LPS. In addition I examined the binding (by immunoblotting) of some human IgG preparations to LPS from the same collection of bacteria

Bacteria (table 5.1)

Ten blood-culture isolates were selected to represent the range and relative frequency of Gram-negative bacilli isolated from blood cultures in the clinical bacteriology laboratory in Department of Medical Microbiology. Identification of these organisms was checked using API20E (Bio Merieux). One strain (8b 226) which had been labelled as a *Klebsiella* was found to be an *E. coli*, and therefore a further *Klebsiella* strain (9b26) was selected. When Polyacrylamide gel electrophoresis was performed the strain of *Proteus* was found to have an unusual pattern (see results) and therefore a second *Proteus* strain (9b24) was selected. O typing of the *E. coli* strains was subsequently performed by the Division of Enteric Pathogens, Central Public Health Laboratory, Colindale, London. The strains are listed in table 5.1 a.

Rough strains of *E. coli* used in this chapter (table 5.1 b) were obtained from the MPRL collection.

MAbs

Most of the MAbs used in this chapter were bulk purified preparations. Their specificities (as determined by polymyxin-LPS ELISA) and concentration are listed in table 5.2.

Table 5.1 Bacteria used in chapter 5

a: Blood culture isolates

Number	Reference letter (used in figures)	Identification
8b 226	a	*E. coli* 0:18 K5
8b 239	b	*Proteus sp.*
8b 257	c	*Enterobacter cloacae*
8b 273	d	*Serratia sp.*
8b 275	e	*E. coli* 0:75 K5
8b 281	f	*E. coli* 0:18 K5
8b 300	g	*Pseudomonas aeruginosa*
8b 305	h	*Pseudomonas aeruginosa*
8b 316	i	*E. coli* 0:1 K1
8b 317	j	*E. coli* 0:6
9b 24	k	*Proteus sp.*
9b 26	l	*Klebsiella pneumoniae*

b: Standard core types of *E. coli*

	Reference numbers		Origin
R1	MPRL 1383	HF4704	Dr Nils Carlin, Sweden
R2	MPRL 1908	F576	Dr G Schmidt, Germany
R3	MPRL 1909	F653	Dr G Schmidt, Germany
R4	MPRL 1382	F2513	Dr Nils Carlin, Sweden
K12	MPRL 1303	2131	Dr Nils Carlin, Sweden
B	MPRL 1900	2156	Dr G Schmidt, Germany

Table 5.2 Characteristics of purified monoclonals used in chapter 5.

MAb Name	Isotype	concentration (mg/ml)	ELISA specificity[a]
J5 4 45.6	IgG3	5.2	J5
H5 13.23	IgG3	9.6	Rc
SZ 27 150.3	IgG2a	8.9	Rc
H1 61.1	IgG1	4.8	Rc
SZ 27 19.16.7	IgG2a	10.3	Rc
H4 351.18	IgG2a	10.3	Rc
SZ 5 28.4.4	IgG2b	2.5	Rc
H7 41.76	IgG1	8.1	Rc
SZ 30 4.2.8	IgM	3.5	Lipid A
SZ 27 193.3	IgM	4.5	Lipid A
Rc 878 2.51.9	IgA	7.3	Rc
SZ 43 27.7.3	IgG	?	Ra

[a] ELISA specificity defined as the smallest LPS chemotype of *Salmonella* or *E. coli* with which the MAb would react in polymyxin-LPS ELISA.

Whole-cell ELISA

Overnight shaken broth cultures were washed and resuspended in normal saline. The concentration of bacteria was determined using an improved Neubauer counting chamber. A typical count was 2 x 10^9/ml. The bacterial suspensions were then diluted in coating buffer to a concentration of 10^7/ml, 100 µl per well added to NUNC polysorb strips, and the strips incubated at 4°C overnight. The strips were then washed four times, and 100 µl per well of post-coating buffer added. After incubation overnight once more at 4°C the plates were again washed four times and stored at -20°C.

Purified MAbs were diluted 1 in 2500 in ELISA diluent without albumin, and 100 µl volumes placed in duplicate wells and incubated for 1 h at 37°C. Plates were then washed four times and 100µl of urease-conjugated sheep-anti-human-IgG (Sera-lab, diluted 1 in 500 in ELISA diluent without albumin) was added and incubated for a further 60 min at 37°C, then washed three times and rinsed five time in distilled water. Urease substrate (100 µl: Sera-lab) was added and left at room temperature for 60 minutes, and the OD read at 590 nm.

Flow Cytometry

Bacteria from overnight broth cultures were washed in PBS and resuspended to give an OD of 0.5 at 525nm. Using Eppendorf tubes, 1 ml volumes were centrifuged and the bacteria resuspended in 1 ml PBS containing a 1 in 100 dilution (50 - 100 µg/ml) of purified MAb. After incubation for 1 hour at 37°C the bacteria were washed twice in

PBS and resuspended in 1 ml of a 1 in 100 dilution of sheep FITC-conjugated anti-mouse IgG (ICN) in PBS and incubated for a further hour at 37°C. The bacteria were then washed twice more and resuspended in 1 ml of PBS containing 0.5% formaldehyde. Prepared samples were diluted 1 in 50 in PBS and analysed using an EPICS "C" (Coulter Electronics) flow cytometer equipped with a 5 watt argon ion laser, operating at 500 mW and exciting and 488 nm. Cells were passed through the beam at approximately 500 per second from a standard 76 µm flow cell tip. Background noise and clumps of cells were excluded by a gate on the log forward angle light scatter. Cells stained with FITC conjugate, but with no primary antibody, provided a background staining level, set at 1% ± 0.5% by adjusting the voltage applied to the green fluorescence log (GFL) photomultiplier tube. A total of 50,000 cells were analysed from each sample and the percentage of cells exhibiting positive staining was calculated by the EPICS "Stat pack" programme.

Co-Agglutination

Calbiochem "Pansorbin" *Staphylococcus aureus* cells (lot 745004) were a gift from Dr H Young of the Department of Medical Microbiology. It consisted of a milky suspension of *S. aureus* cells. Following the manufacturers instructions, 1 ml volumes were washed 3 times in co-agglutination buffer (PBS containing 0.1% sodium azide, pH8). The cells were then resuspended in the same buffer containing 2 mg/ml of MAb, mixed, and incubated at room temperature for 15 minutes. Cells were then washed a further 3 times in co-agglutination buffer. A control suspension treated in the same way but with no added MAb was also prepared.

To test binding to bacteria, a colony from a nutrient agar plates was suspended in a drop of PBS on a dark tile. A drop (40 µl) of co-agglutination reagent was added, mixed by rotation, and inspected for the development of agglutination.

"PHAST" system

The "PHAST" system is a commercial SDS-PAGE and immunoblotting method using small (usable area 2 inch square) factory made gels. Because the gels are mass-produced they are said to give more reproducible results and, because they are small, running the gel takes about 1 hour. The whole process including blotting, reacting with antibody and staining can easily be completed within one day. The washing and staining of gels and blots can be run automatically, further improving reproducibility and reducing hands-on time. A "PHAST" system was obtained on loan from Pharmacia for a trial.

Gels of various concentrations, including gradient gels, are available. The gel contains no buffer, and strips containing of buffer (with or without SDS) are used . The gel lies horizontally in the apparatus, and the samples of material to be separated are placed on the surface of the gel, instead of being placed in wells. There is considerable literature on the use of this system for analysis of proteins, but no information was available relating to the analysis of LPS.

INTERACTION OF MABS AND BACTERIA IN IMMUNOBLOTS AND WHOLE-CELL ELISA

Silver stain of LPS from blood culture isolates of Gram-negative bacilli.

Figure 5.1 shows a silver stained polyacrylamide gel of LPS prepared by proteinase K digestion from the first 10 isolates chosen. All but one produced a "ladder" pattern, as expected from wild-type Gram-negative bacilli. Strain 8b 239 (lane b; *Proteus* sp.) had material in the region where high molecular weight bands of LPS would be expected, but these did not resolve into distinct bands. The two strains of *E. coli* 0:18 (lanes a and f) produced almost identical ladder patterns, consistent with the idea that the repeating O-antigen unit was the same in these two isolates. The two strains of *Pseudomonas aeruginosa* (lanes g and h) also produced patterns that were similar to one another. Two further isolates were then chosen, as discussed above, and a silver-stained gel of the series of 12 strains made (frontispiece). The second *Proteus* sp. gave a similar pattern to the first, with no distinct ladder pattern, and therefore it was concluded that this appearance must be usual, or at least quite common, in *Proteus* isolates. The *Klebsiella pneumoniae* isolate did give a typical ladder pattern, though this is not well seen in the photograph because the gel has been allowed to develop too long.

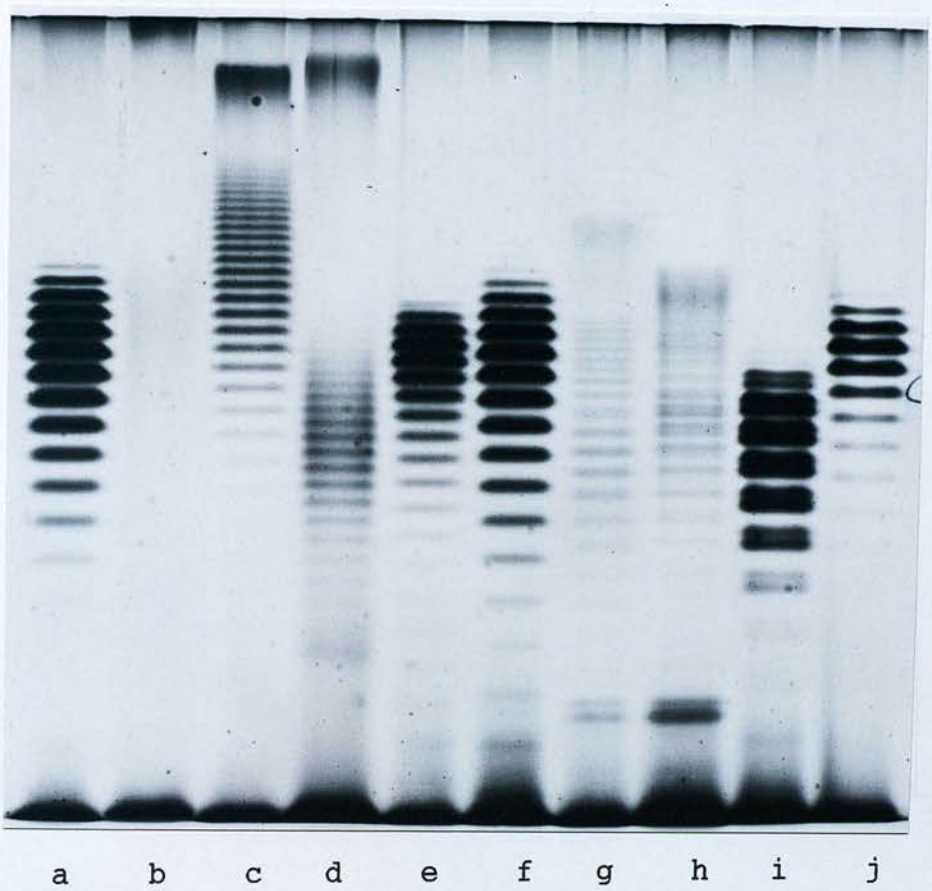


Figure 5.1 Silver-stained polyacrylamide gel of LPS (proteinase K digests) from 10 blood-culture. Lane a = 8b226 (*E. coli*); b = 8b239 (*Proteus sp*); c = 8b257 (*Enterobacter cloacae*); d = 8b273 (*Serratia sp.*); e = 8b275 (*E. coli*); f = 8b281 (*E. coli*); g = 8b300 (*Pseudomonas aeruginosa*); h = 8b305 (*Pseudomonas aeruginosa*); i = 8b316 (*E. coli*); j = 8b317 (*E. coli*).

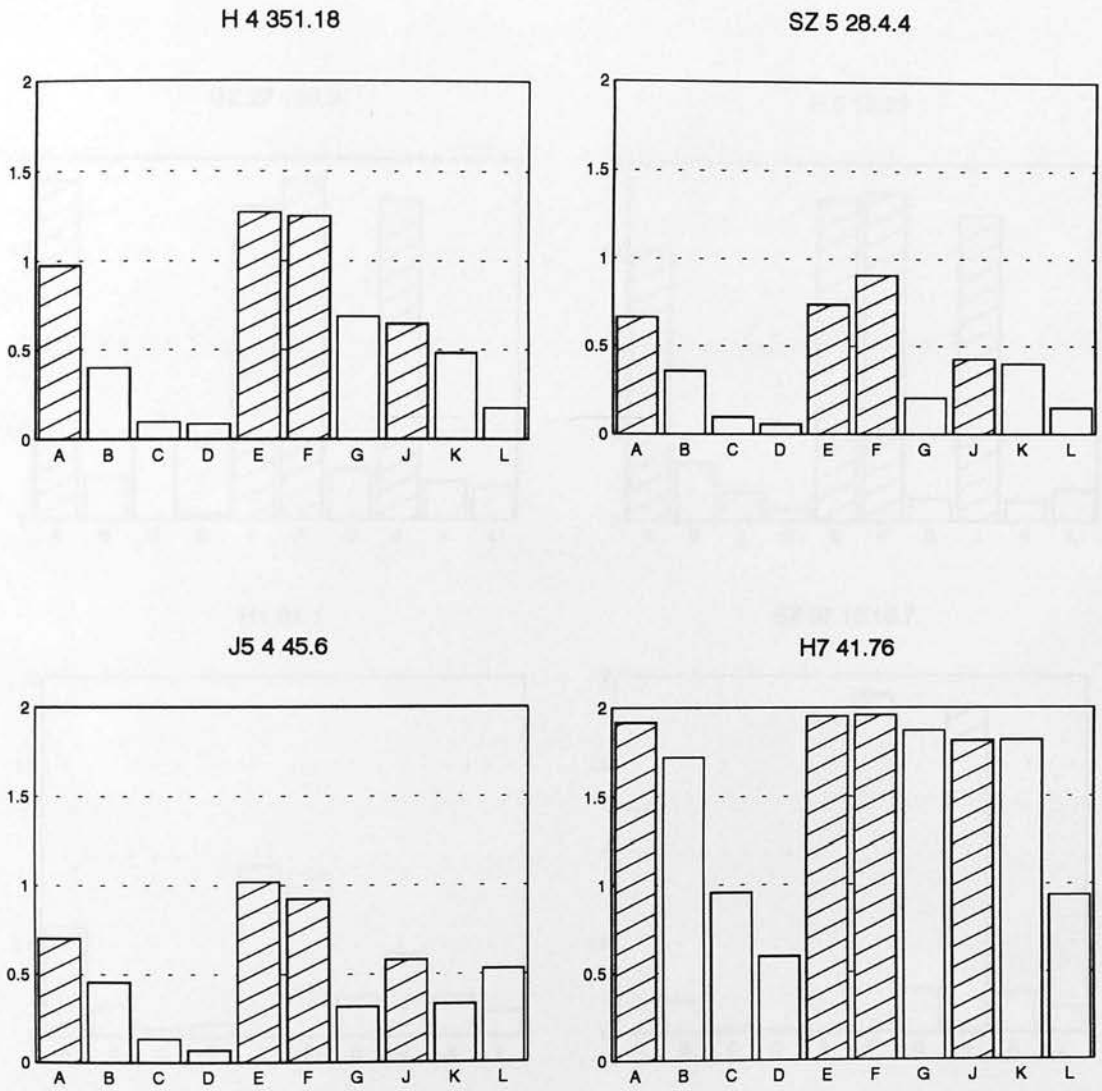


Figure 5.2 Reactivity in ELISA of 4 MAbs with panel of 10 blood culture isolates. A = 8b226; B = 8b239; C = 8b257; D = 8b273; E = 8b275 F= 8b281; G = 8b300; J = 8b317; K = 9b24; L = 9b26. Cross-hatching indicates E.coli strains. Results are mean of two OD readings.

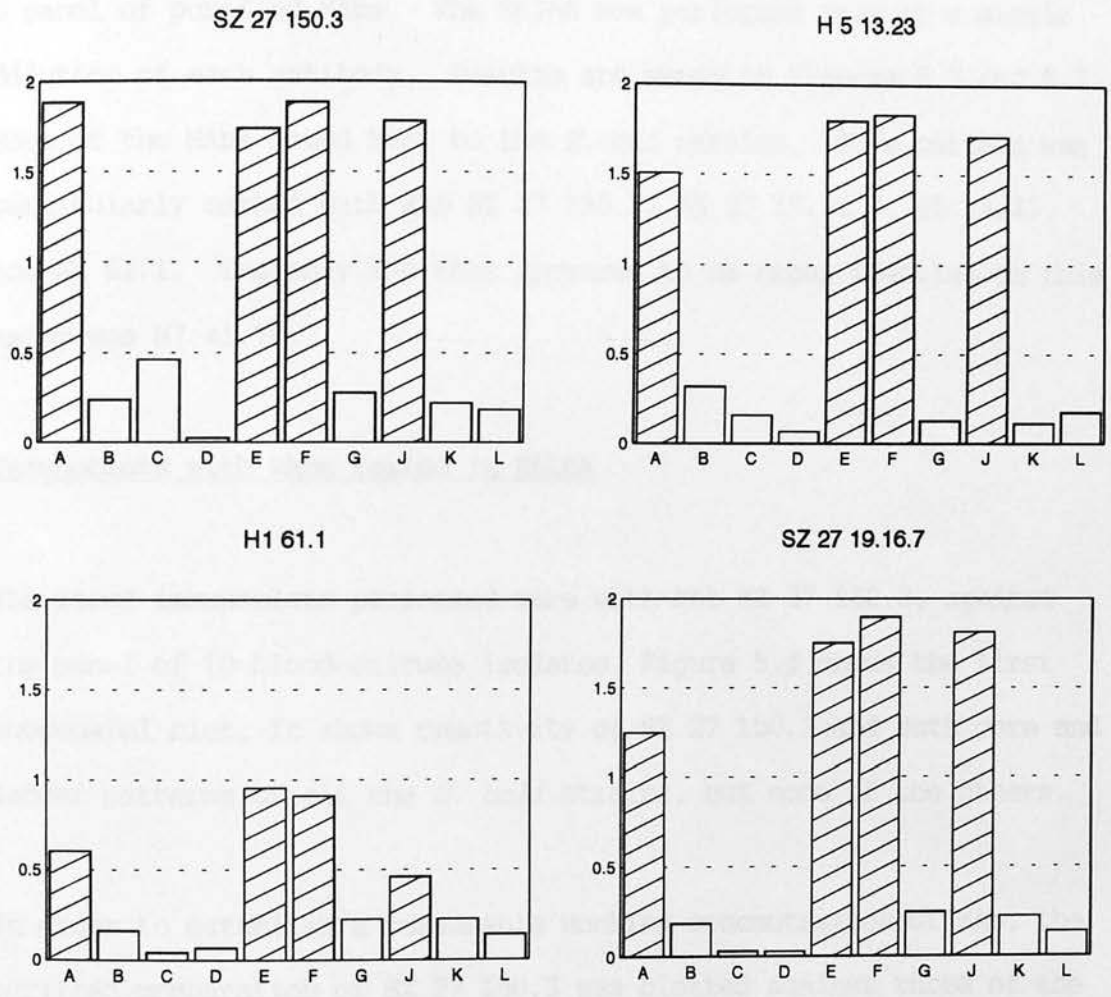


Figure 5.3 Reactivity in ELISA of 4 MAbs with panel of 10 blood culture isolates. A = 8b226; B = 8b239; C = 8b257; D = 8b273; E = 8b275 F= 8b281; G = 8b300; J = 8b317; K = 9b24; L = 9b26. Cross-hatching indicates E.coli strains. Results are mean of two OD readings.

Whole cell ELISA

Whole-cell ELISA was performed with 10 of the 12 isolates (omitting one of the *P. aeruginosa* strains and one of the *E. coli* strains) and a panel of purified MAbs. The ELISA was performed only at a single dilution of each antibody. Results are shown in figures 5.2 and 5.3. Most of the MAbs bound best to the *E.coli* strains. This pattern was particularly marked with MAb SZ 27 150.3, SZ 27 19.16.7, H5 13.23, and H1 61.1. The only MAb that appeared to be cross-reactive on this assay was H7 41.76.

Immunoblots with MAbs tested in ELISA

The first immunoblots performed were with MAb SZ 27 150.3, against the panel of 10 blood-culture isolates. Figure 5.4 shows the first successful blot. It shows reactivity of SZ 27 150.3 and both core and ladder patterns of all the *E. coli* strains, but none of the others.

In order to establish a reasonable working concentration of MAb, the purified preparation of SZ 27 150.3 was blotted against three of the *E. coli* strains, at dilutions of 1 in 200 (as used above), 1 in 400, and 1 in 800. As shown in figure 5.5, the blot gave a much darker result on this occasion. It was apparent however that the bands produced with the 1 in 800 dilution were less dark than those produced at 1 in 400, and that the background was darker at 1 in 200. It was decided to use a 1 in 500 dilution of purified MAb (equivalent to approximately 18 µg/ml) for future experiments.

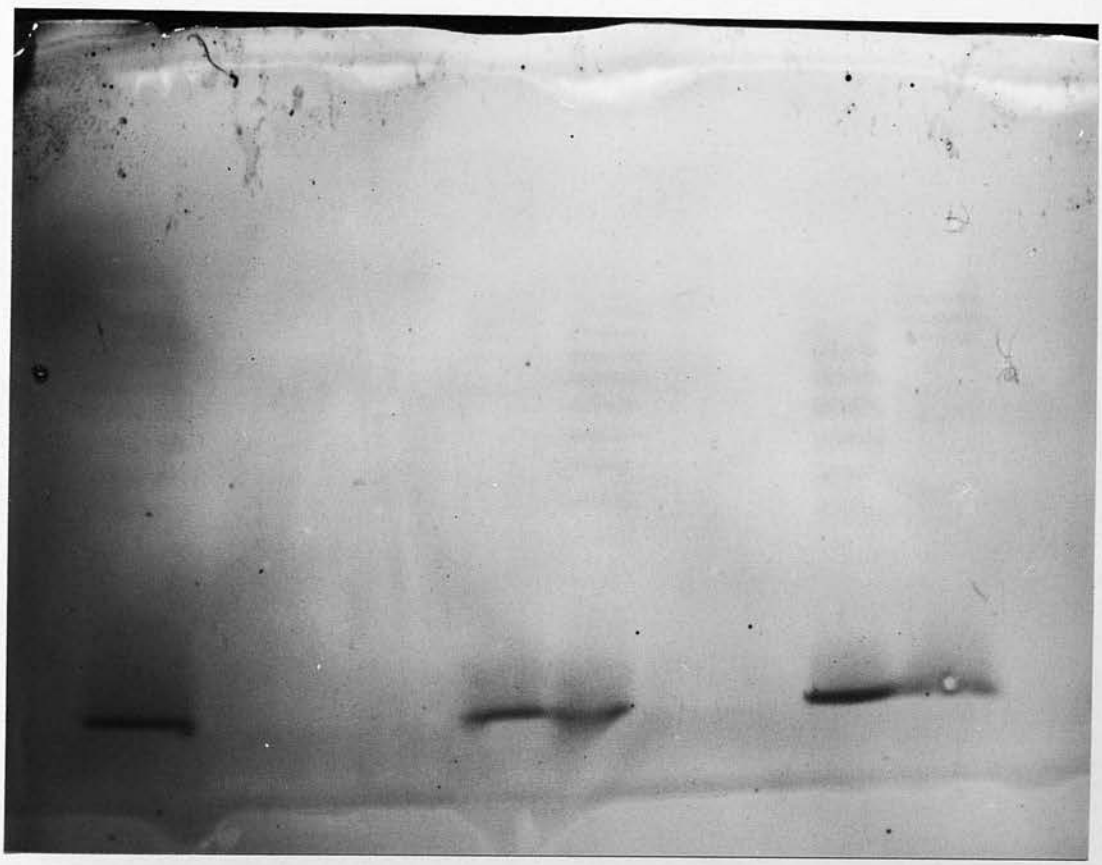


Figure 5.4 Immunoblot of MAb SZ 27 150.3 against proteinase K digests of 10 blood culture isolates. The antibody was purified preparation, diluted 1 in 200, = 44 µg/ml. Lane a = 8b226 (*E. coli*); b = 8b239 (*Proteus sp*); c = 8b257 (*Enterobacter cloacae*); d = 8b273 (*Serratia sp.*); e = 8b275 (*E. coli*); f = 8b281 (*E. coli*); g = 8b300 (*Pseudomonas aeruginosa*); h = 8b305 (*Pseudomonas aeruginosa*); i = 8b316 (*E. coli*); j = 8b317 (*E. coli*).

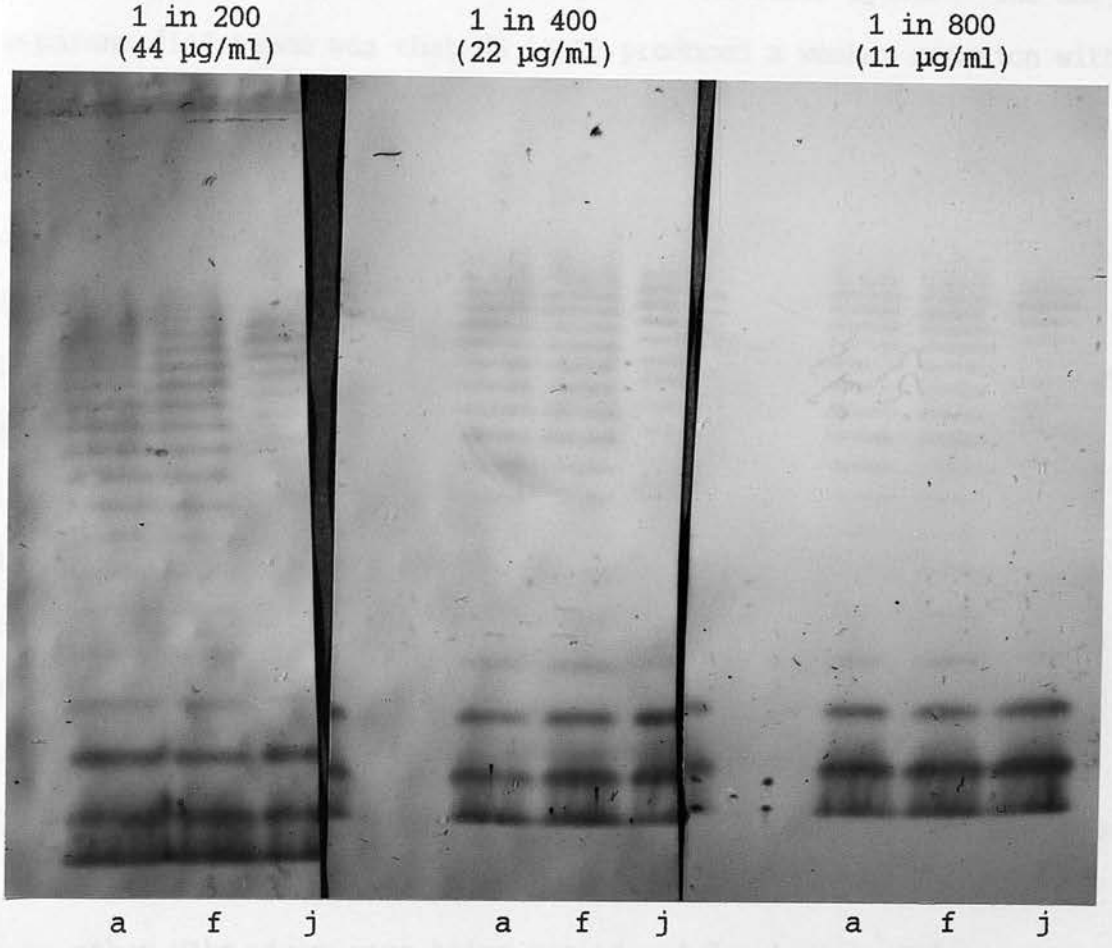


Figure 5.5 Immunoblot of MAb SZ 27 150.3 (purified preparation) against proteinase K digests of three *E.coli* strains at three dilutions of MAb. Lane a = 8b 226; f = 8b 281, and j = 8b 317. Dilutions (approximate concentration) of MAb shown above.

Immunoblots were made with of each of the antibodies tested in the ELISA against LPS extracted by proteinase K digestion of the panel of 12 organisms. Examples are shown in figures 5.6 - 5.8. H5 13.23 (figure 5.6) and SZ 27 19.16.7 (not shown) gave similar patterns to that produced by SZ 27 150.3 (figure 5.4), with ladder patterns for the *E.coli* strains but no reactivity with the other species. The only apparent difference was that H5 13.23 produced a weaker reaction with 8b 317 (*E. coli* O:6) than did the other two. H4 351.18 (figure 5.7) and H7 41.76 (figure 5.8) reacted only with the fastest moving band of some of the *E. coli* strains (strains 8b 226, 8b 275, and 8b281: lanes a, e, and f). J5 4 45.6 , H1 16.1 & SZ 5 28.4.4 produced completely negative blots. Blots with these last three were repeated for three of the *E. coli* strains alone, with SZ 27 19.16.7 as a control. A single gel with four sets of bands was blotted and the blot cut into four pieces which were reacted simultaneously with one of the four MAbs. SZ 29 19.16.7 produced a ladder pattern as before, but the other three MAbs again produced negative results (not shown).

Immunoblots with other MAbs against the panel of 12 clinical isolates

Four other MAbs which were being considered for larger-scale production and more detailed investigation by Sandoz were blotted against the panel of 12 clinical isolates.

SZ 27 193.3 was an IgM MAb which bound to most of the LPS chemotypes used in LPS-polymyxin ELISA, including lipid A, and was therefore thought provisionally to be an anti-lipid A antibody. In the

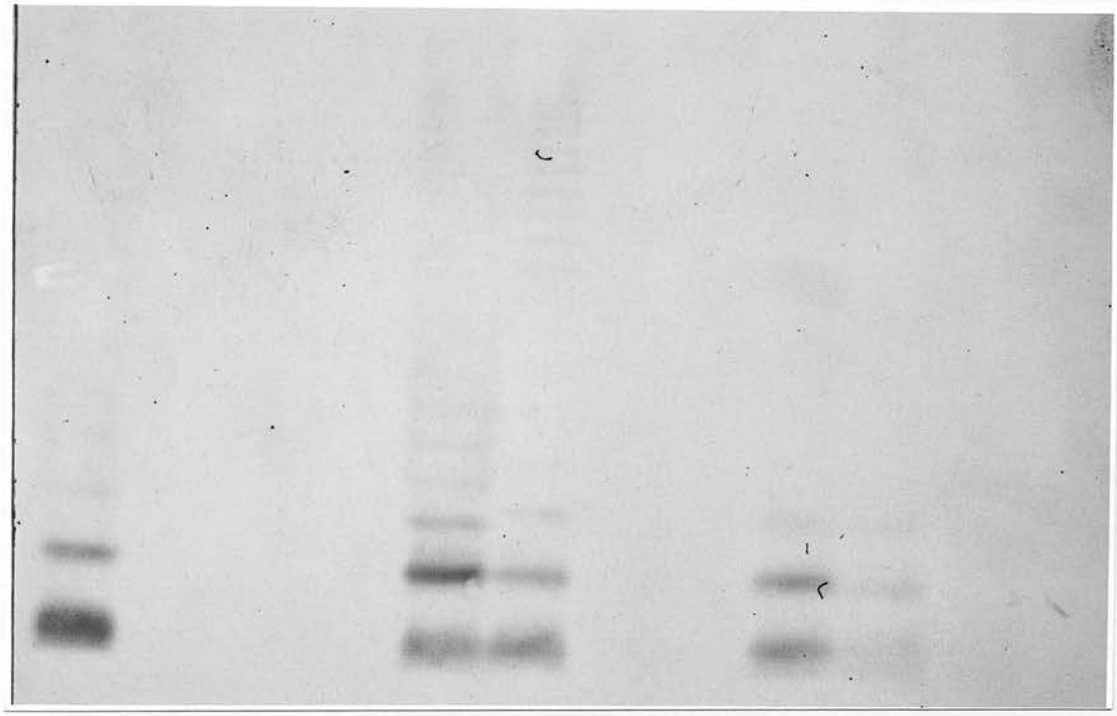


Figure 5.6 Immunoblot of H5 13.23 against proteinase K digests of 12 blood culture isolates. Lane a = 8b226 (*E. coli*); b = 8b239 (*Proteus sp*); c = 8b257 (*Enterobacter cloacae*); d = 8b273 (*Serratia sp.*); e = 8b275 (*E. coli*); f = 8b281 (*E. coli*); g = 8b300 (*Pseudomonas aeruginosa*); h = 8b305 (*Pseudomonas aeruginosa*); i = 8b316 (*E. coli*); j = 8b317 (*E. coli*); k = 9b24 (*Proteus sp.*); l = 9b 26 (*Klebsiella pneumoniae*).

Figure 5.7 Immunoblot of H4 351.18 against proteinase K digests of 12 blood culture isolates. Lane a = 8b226 (*E. coli*); b = 8b239 (*Proteus sp*); c = 8b257 (*Enterobacter cloacae*); d = 8b273 (*Serratia sp.*); e = 8b275 (*E. coli*); f = 8b281 (*E. coli*); g = 8b300 (*Pseudomonas aeruginosa*); h = 8b305 (*Pseudomonas aeruginosa*); i = 8b316 (*E. coli*); j = 8b317 (*E. coli*); k = 9b24 (*Proteus sp.*); l = 9b 26 (*Klebsiella pneumoniae*).

a b c d e f g h i j k l

Figure 5.8 Immunoblot of H7 41.76 against proteinase K digests of 12 blood culture isolates. Lane a = 8b226 (*E. coli*); b = 8b239 (*Proteus sp*); c = 8b257 (*Enterobacter cloacae*); d = 8b273 (*Serratia sp.*); e = 8b275 (*E. coli*); f = 8b281 (*E. coli*); g = 8b300 (*Pseudomonas aeruginosa*); h = 8b305 (*Pseudomonas aeruginosa*); i = 8b316 (*E. coli*); j = 8b317 (*E. coli*); k = 9b24 (*Proteus sp.*); l = 9b 26 (*Klebsiella pneumoniae*).

a b c d e f g h i j k l

Figure 5.9 Immunoblot of SZ 27 193.3 against proteinase K digests of 12 blood culture isolates. Lane a = 8b226 (*E. coli*); b = 8b239 (*Proteus sp*); c = 8b257 (*Enterobacter cloacae*); d = 8b273 (*Serratia sp.*); e = 8b275 (*E. coli*); f = 8b281 (*E. coli*); g = 8b300 (*Pseudomonas aeruginosa*); h = 8b305 (*Pseudomonas aeruginosa*); i = 8b316 (*E. coli*); j = 8b317 (*E. coli*); k = 9b24 (*Proteus sp.*); l = 9b 26 (*Klebsiella pneumoniae*).

immunoblot (figure 5.9) it bound to material in a regular band across all the tracks which corresponded to the interface between the stacking and separating gel. No clear band could be seen in the region where bands containing LPS (and therefore lipid A) were expected.

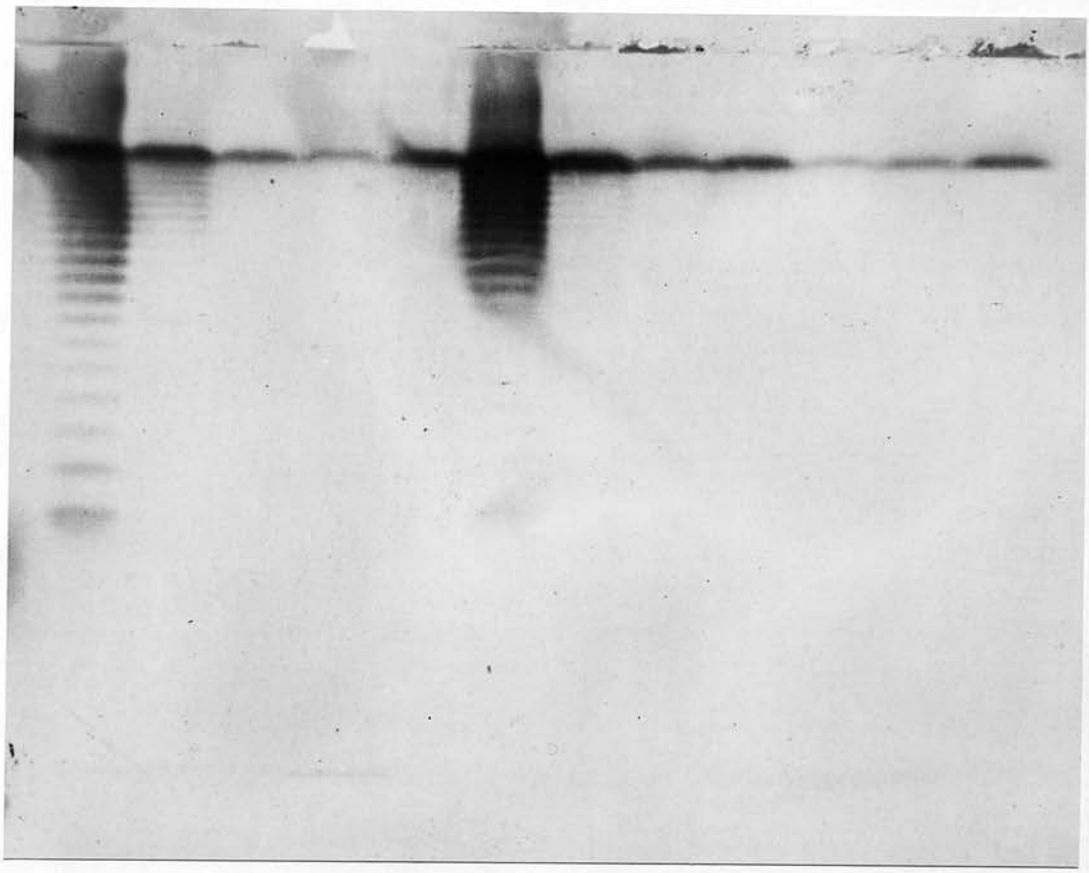
SZ 30 4.2.8, like SZ 27 193.3, was an IgM which bound to a wide range of LPS and to lipid A in LPS-polymyxin ELISA. Particularly strong binding to *E. coli* 0:18 strains had been noted. In the immunoblot (figure 5.10) it bound to material at the top of the separating gel, as seen with SZ 27 193.3. It also reacted with the upper part of the "ladder" pattern of both the *E. coli* 0:18 strains.

Rc 878 2.51.9 was an IgA MAb which bound with a limited range of rough and smooth LPS in polymyxin-LPS ELISA. In the immunoblot (figure 5.11) it bound to the leading band of the *E. coli* isolates, and to the material at the top of the stacking gel.

SZ 43 27.7.3 was an IgG which bound to a wide range of LPS, but particularly to *E. coli* R1 and some smooth *E. coli* in LPS-polymyxin ELISA. In the immunoblot (figure 5.12) it bound to the leading band of all the *E. coli* strains, but not to the other species.

<u>Binding to the "ladder" pattern versus binding to a single band in immunoblots.</u>

The fastest moving band seen in the silver stain is thought to consist of the complete LPS-core structure, including Lipid A, but



a b c d e f g h i j k l

Figure 5.10 Immunoblot of SZ 30 4.2.8 against proteinase K digests of 12 blood culture isolates. Lane a = 8b226 (*E. coli*); b = 8b239 (*Proteus sp*); c = 8b257 (*Enterobacter cloacae*); d = 8b273 (*Serratia sp.*); e = 8b275 (*E. coli*); f = 8b281 (*E. coli*); g = 8b300 (*Pseudomonas aeruginosa*); h = 8b305 (*Pseudomonas aeruginosa*); i = 8b316 (*E. coli*); j = 8b317 (*E. coli*); k = 9b24 (*Proteus sp.*); l = 9b 26 (*Klebsiella pneumoniae*).

a b c d e f g h i j k l

Figure 5.11 Immunoblot of Rc 878 2.51.9 against proteinase K digests of 12 blood culture isolates. Lane a = 8b226 (*E. coli*); b = 8b239 (*Proteus sp*); c = 8b257 (*Enterobacter cloacae*); d = 8b273 (*Serratia sp.*); e = 8b275 (*E. coli*); f = 8b281 (*E. coli*); g = 8b300 (*Pseudomonas aeruginosa*); h = 8b305 (*Pseudomonas aeruginosa*); i = 8b316 (*E. coli*); j = 8b317 (*E. coli*); k = 9b24 (*Proteus sp.*); l = 9b 26 (*Klebsiella pneumoniae*).

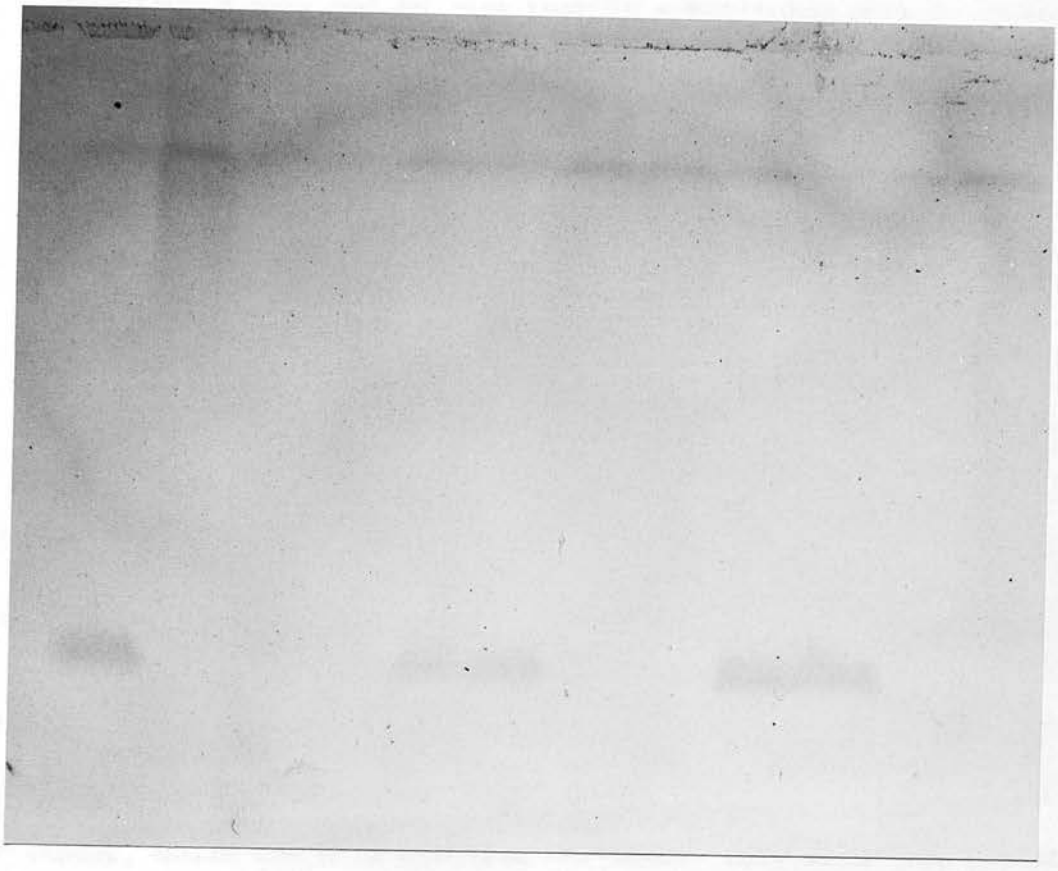


Figure 5.12 Immunoblot of SZ 43 27.7.3 against proteinase K digests of 12 blood culture isolates. Lane a = 8b226 (*E. coli*); b = 8b239 (*Proteus sp*); c = 8b257 (*Enterobacter cloacae*); d = 8b273 (*Serratia sp.*); e = 8b275 (*E. coli*); f = 8b281 (*E. coli*); g = 8b300 (*Pseudomonas aeruginosa*); h = 8b305 (*Pseudomonas aeruginosa*); i = 8b316 (*E. coli*); j = 8b317 (*E. coli*); k = 9b24 (*Proteus sp.*); l = 9b 26 (*Klebsiella pneumoniae*).

with no O antigen substitution. The next band is thought to consist of LPS with only a single O-antigen unit, and each subsequent band consists of molecules with one more O-antigen repeating unit[180]. The two different patterns of binding to individual *E. coli* LPS could therefore be explained by some of the MAbs (eg H4 351.18) binding only to unsubstituted core, while others (eg SZ 27 150.3) bind both to unsubstituted core and to core that is substituted with O antigen. To compare the patterns of binding more clearly a gel was prepared using a single broad track of LPS (proteinase K digest) from 8b 281 (*E. coli* O:18). Half of this gel was blotted onto nitrocellulose and cut into strips which were then reacted with SZ 27 150.3, H5 13.23, SZ 43 27.7.3, and an O:18 specific monoclonal ES 184.4.1. This confirmed (figure 5.13) that the fastest moving band seen on the silver-stained gel corresponds to the single positive band seen in the immunoblot with SZ 43 27.7.3, and that this band does not react with the O-specific MAb. SZ 27 150.3 and H5 13.23 react with this fast moving band and also with some of the slower-moving bands which are seen in the silver stain and which react with the O-specific MAb. SZ 27 150.3 and H5 13.23 react preferentially with the lower part of the ladder, while the O:18 specific MAb reacts more with the higher bands. This difference presumably reflects the greater proportion of O antigen in the higher bands and the greater proportion of core material in the lower bands.

Immunoblots with standard R types

Slight but consistent differences in binding to different *E. coli* isolates were observed in both ELISA and immunoblots. One possible

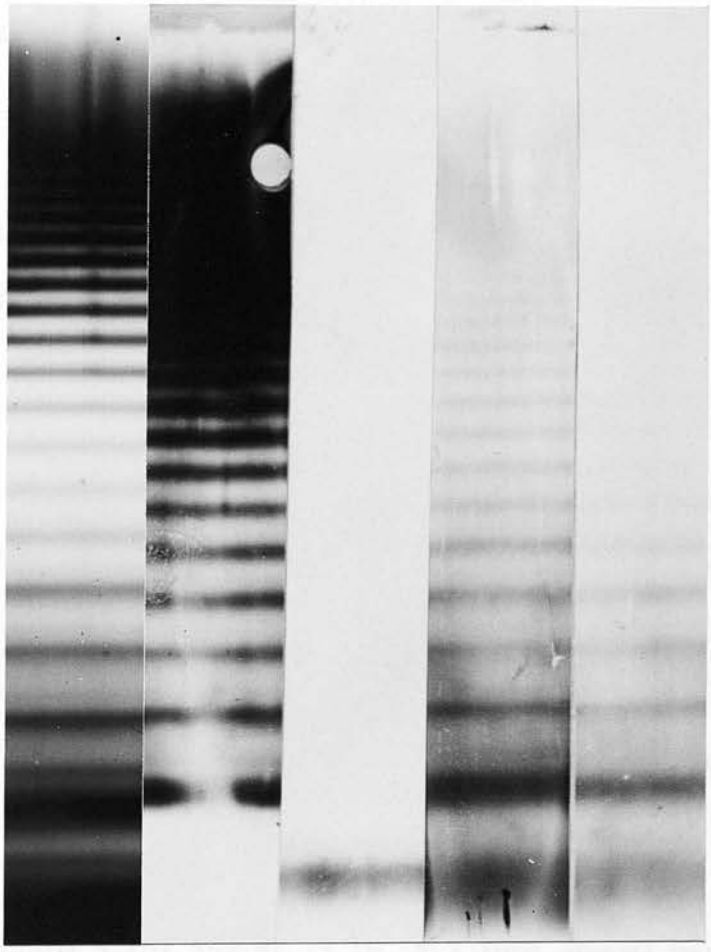


Figure 5.13 Silver stain and immunoblots of proteinase K preparations of LPS from *E. coli* O:18 (strain 8b281). Lanes: 1 = silver stain; 2-5 show immunoblots obtained with MAbs ES 184.4.1 (O:18 specific), H4 351.8, H5 13.23, and SZ 27/150.3.

explanation for this would be that the LPS-core of the wild-type strains used differed. I therefore decided to examine the binding of some MAbs to the range of known core-types of *E. coli* and *Salmonella* in immunoblots, together with two of the blood culture isolates of *E. coli* which served as positive controls. .ig figure 5.13

In the first of these experiments (figures 5.14 and 5.15) the strain thought to be K12 was found subsequently to be a *Salmonella* Ra mutant. Note also that track h of the silver-stained gel (figure 5.14) contained LPS from *E. coli* 8b316, while *E. coli* 8b317 LPS was used at the equivalent position in the subsequent blots (figure 5.15).

The silver stain (figure 5.14) of the rough LPSs showed, as expected, bands corresponding to the leading (unsubstituted core) band of the smooth LPSs. In addition however the silver stain showed slower moving material which did not resolve clearly into bands. On some occasions, particularly in some of the blots, discrete bands could be seen in this material.

SZ 27 150.3 (figure 5.15) and SZ 27 19.16.7 (not shown) reacted with all the rough LPSs, and with both unsubstituted and substituted core of the two blood culture isolates. H5 13.23 reacted with substituted and unsubstituted core of the blood culture isolates, and with *E. coli* R1, R2 and K12, and with the *Salmonella* rough mutant. H4 351.18 and H7 41.76 both reacted with *E. coli* R2 and with the *Salmonella* rough mutant, and with the unsubstituted core band from *E. coli* 8b226 but not 8b317. Binding to the diffuse slow-moving material seen in the silver stain corresponded with binding to the core band of the

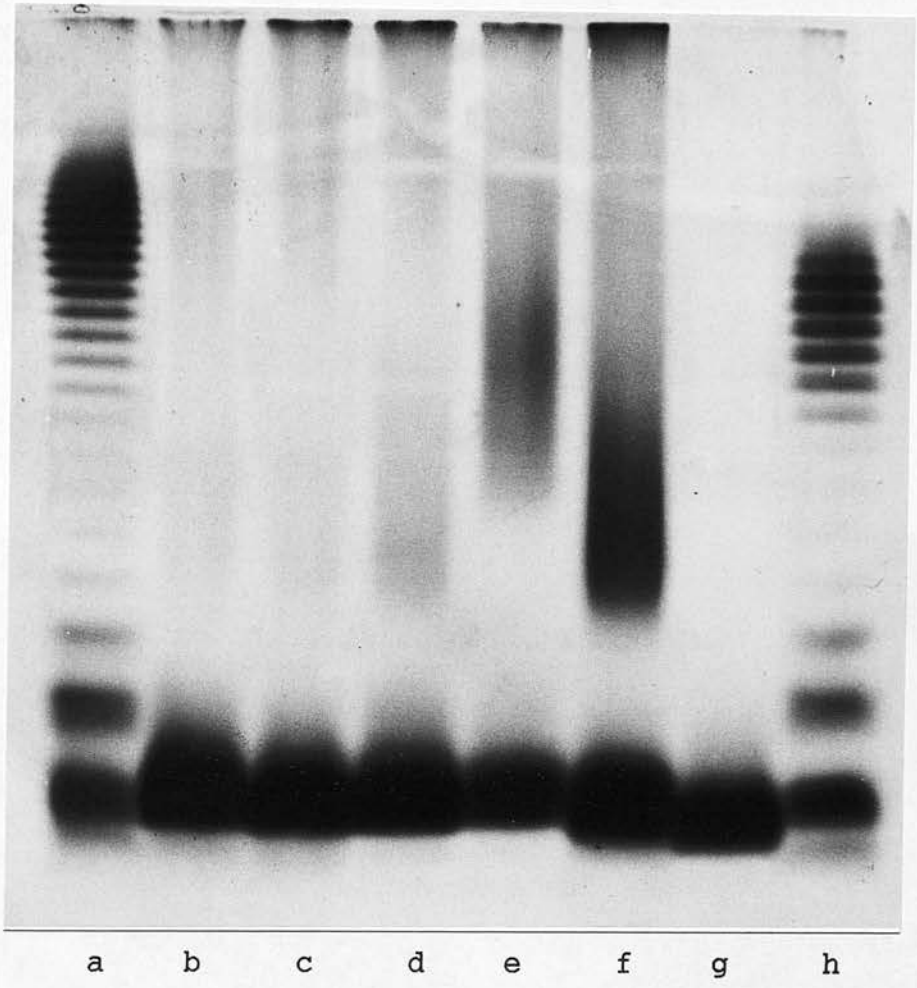


Figure 5.14 Silver-stained polyacrylamide gel of LPS (proteinase K digests) from rough and smooth *E. coli* or *Salmonella*. a = 8b 226; b = R1; c = R2; d = R3; e = R4; f = *Salmonella*; g = B; h = 8b 316.

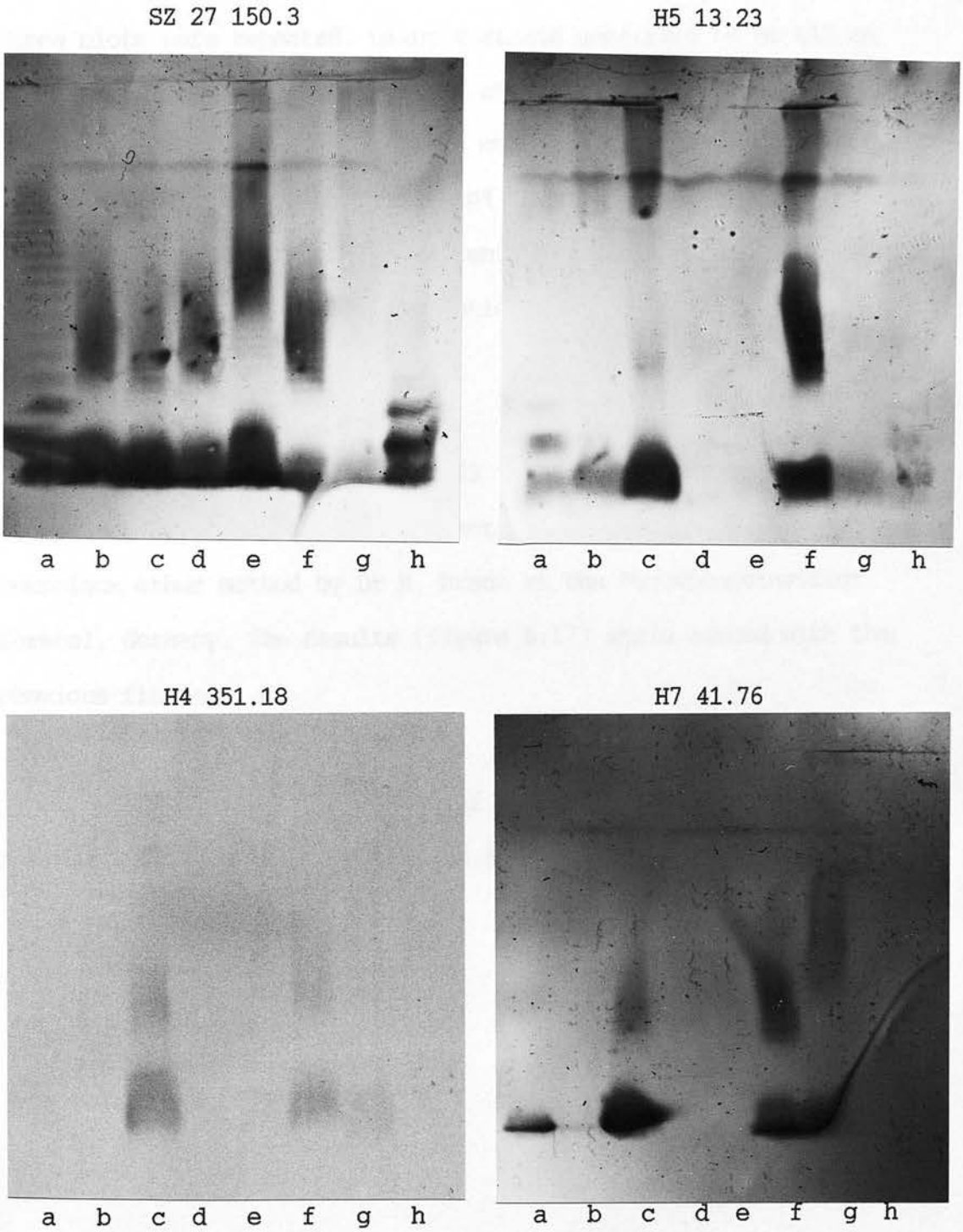


Figure 5.15 Immunoblot of four MAbs against LPS (proteinase K digests) from rough and smooth *E. coli* or *Salmonella*. Lane a = *E. coli* 8b226; b = R1; c = R2; d = R3; e = R4; f = *Salmonella* rough mutant; g = *E. coli* B; h = *E. coli* 8b317.

appropriate track.

These blots were repeated, using a strain confirmed to be K12 on biochemical and phage testing. *E. coli* B was omitted from these blots in order that the four blots could be run simultaneously on the two available gel tanks, each set of bands occupying half of one gel. Results (Figure 5.16) were consistent with the previous experiment. K12 reacted in these blots an identical way to the *Salmonella* rough mutant in the previous blots.

The blots of SZ 27 150.3, H5 13.23 and H7 41.76 were then repeated using rough LPS preparations extracted by the phenol, chloroform, petroleum ether method by Dr H. Brade at the Forschungsinstitut Borstel, Germany. The results (figure 5.17) again agreed with the previous findings.

Table 5.3 summarises the results of immunoblotting with MAbs SZ 27 150.3, SZ 27 19.16.7, H5 13.23, H4 351.18, and H7 41.76.

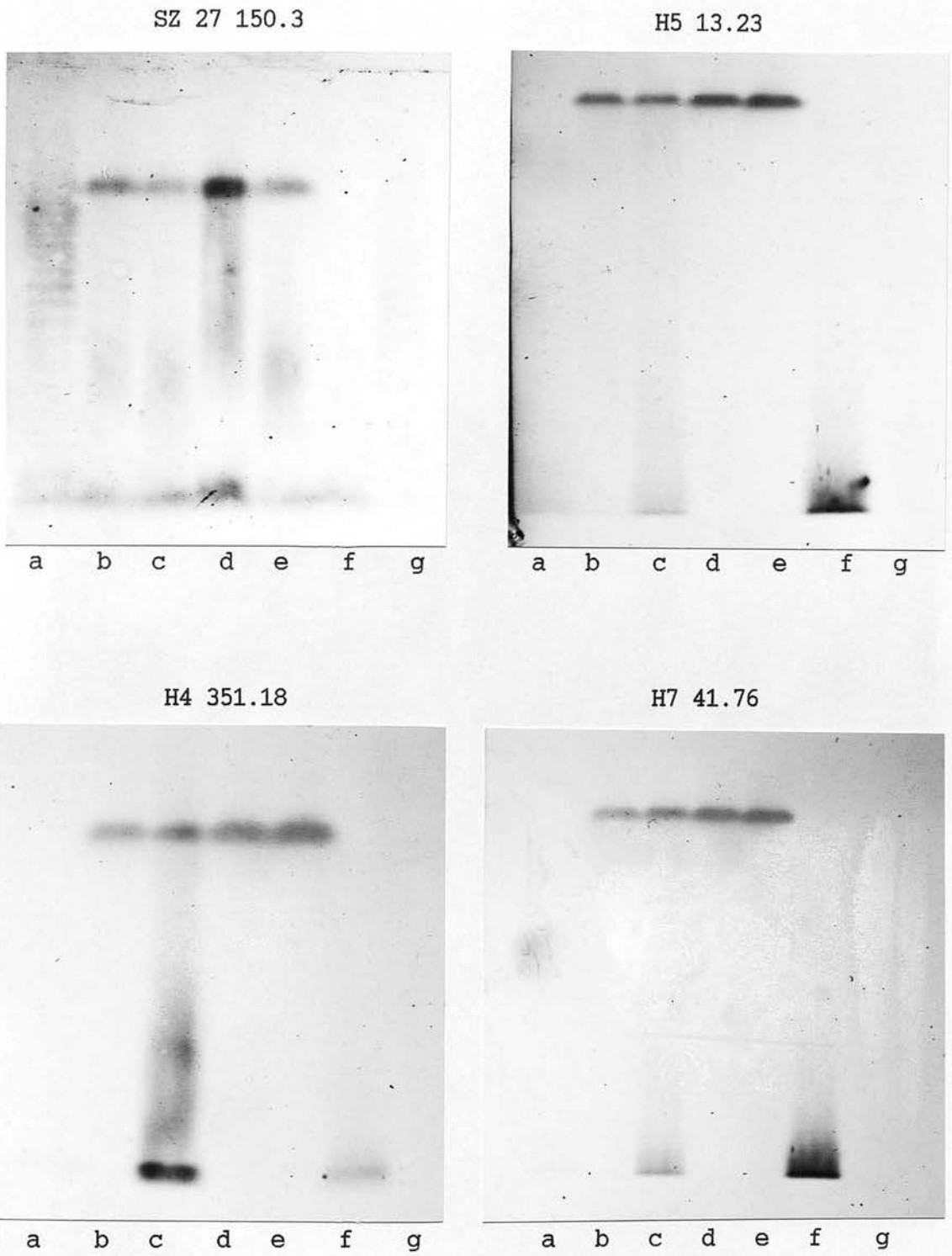


Figure 5.16 Immunoblot of four MAbs against LPS (proteinase K digests) from rough and smooth *E. coli*. Lane a = 8b 226; b = R1; c = R2; d = R3; e = R4; f = K12; g = 8b 317.

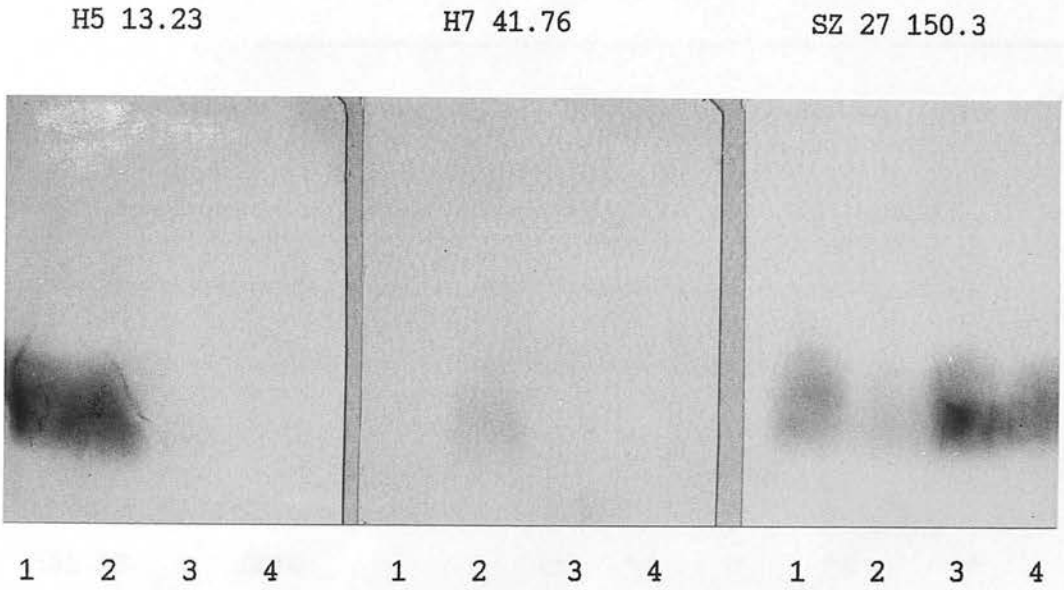


Figure 5.17 Immunoblots of three MAbs against rough *E. coli* LPS. The LPS was prepared by phenol, chloroform, petroleum ether method by Dr H. Brade at the Forschungsinstitut Borstel, Germany. LPS chemotypes were: 1 = R1; 2 =R2; 3 = R3; 4 = R4;.

Table 5.3 Summary of reactivity of purified MAbs with LPS in immunoblots

MAb	Reactivity with LPS from:						
		E. coli					*Salmonella*
	Smooth	R1	R2	R3	R4	K12	
SZ 27 150.3	ladder	+	+	+	+	+	+
SZ 27 19.16.7	ladder	+	+	+	+	+	+
H5 13.23	ladder	+	++	-	-	++	++
H4 351.18	Core	-	++	-	-	++	++
H7 41.76	Core	-	++	-	-	++	++

Notes. + = weak band; ++ = strong band; ND = not done.

INTERACTION OF MAB AND BACTERIA IN OTHER SYSTEMS

Co-agglutination

Co-agglutination might be a useful rapid way to test the ability of MAbs to bind to bacteria. This approach might be useful either for screening of a large collection of organisms, or for quickly testing clinical isolates to aid in identification or to direct therapy. An attempt was therefore made to produce co-agglutination reagents with MAbs SZ 27 150.3, H5 13.23, and H7 41.76.

The reagent made with H7 41.76 was clumped (auto-agglutinated) and therefore unusable. The H5 13.23 and SZ 27 150.3 reagents were usable initially, and were tested against a small number of *E. coli* strains immediately after production. The preparations were both clumped by suspension of *E. coli* R1, R2, R3, 8b 273, and 8b 281. Clumping occurred within 10 seconds with the rough strains, and was coarse, while with the smooth strains fine clumping was observed after 1-2 minutes. Heating the organisms to 100°C for 15 minutes did not alter the character of the clumping observed. Unfortunately no good negative control organism was tested on that day. Three days later, when suitable negative controls were available, both the remaining preparations had also auto-agglutinated, and could not be tested further.

It was decided not to pursue this approach. It would have been possible to make up co-agglutinating reagents quickly for immediate use, but this would require large quantities of MAb.

Table 5.4 Binding of MAbs to whole *E. coli* as measured in flow cytometry.

E. coli strain	Number (%) of bacteria exhibiting fluorescence above background level with MAb given:		
	SZ 27 150.3	H5 13.23	H7 41.76
R1	40.7	49.8	55.7
R2	25.5	60.6	81.5
R3	21.0	31.0	63.4
R4	29.3	20.2	31.0
8b 281	0[a]	0	0
8b 317	0	0	0

Note [a] 0 = less than background.

Flow Cytometry

The binding of MAbs SZ 27 150.3, H5 13.23 and H7 41.76 to *E. coli* strains R1, R2, R3, R4, 8b 281, and 8b 317 was examined. As shown in table 5.4, all of the MAbs bound to all the rough mutants, in a way that showed less discrimination between the different core types than did the immunoblot. There was no detectable binding to either of the smooth strains.

Other work in progress in the laboratory [165] confirmed that none of these MAbs bound (as determined by flow cytometry) to smooth *E. coli* grown in nutrient broth. Since it appeared that flow cytometry was not immediately useful for studying the binding of MAbs to a wide range of wild-type organisms, nor for discriminating between the different core-types of *E. coli*, I did not pursue it further.

"PHAST" system

Gels were run on the PHAST system with LPS (prepared by aqueous phenol extraction) from *E. coli* 8b 281 (O:18). Tracks containing 2 µg, 0.4 µg, and 0.2 µg LPS were run on 4-15% gradient gels and 12.5% gels, both with SDS-free and with SDS containing buffer, and were silver stained using the standard method for large gels, and using the faster method described by Fomsgaard et al [70]. The SDS-containing buffer strips resulted in completely blank silver stains. SDS-free buffer resulted silver stains with apparently non-specific staining of the front half of the gel, with no bands seen in the expected regions.

An immunoblot was performed using a 12.5% gel and SDS-free buffer. The resulting blot was reacted with MAb SZ 184 2.5.5 (O:18 specific). The result is shown in figure 5.18. Most of the staining is seen in the region where the LPS samples were originally applied, and there is no resolution into bands. It was concluded that LPS had failed to enter the gel, thus explaining the negative silver stains.

IMMUNOBLOTS WITH HUMAN IGG PREPARATIONS

Three human IgG preparations were available for study: a) "Normal" IgG preparation for intravenous injection, prepared from unselected blood donors; b) "Anti-CGL" IgG , prepared in the same way, but from blood donors selected because of high levels of IgG antibody to LPS-core (core glycolipid, CGL) in the polymyxin-LPS-core cocktail ELISA [9], c) "J5" IgG provided by Dr T Calandra [38], prepared from donors immunised with *E. coli* J5.

A polyacrylamide gel was prepared with a single wide track of LPS (prepared by aqueous phenol extraction) from *E. coli* strain 8b 281. A 400 µg sample of LPS was used for the complete gel which is equivalent to 20 normal tracks plus spacers. The gel was run and blotted, and the blot cut into strips which were reacted with varying concentrations of the three IgG preparations as shown in figure 5.19. This showed reactivity with the upper part of the ladder pattern, representing O antigen, by both the "J5" and the "anti-CGL" preparations at the lowest concentration used. "Normal" IgG produced detectable bands in this region only at the highest concentration used. The "anti-CGL" preparation also produced a distinct band

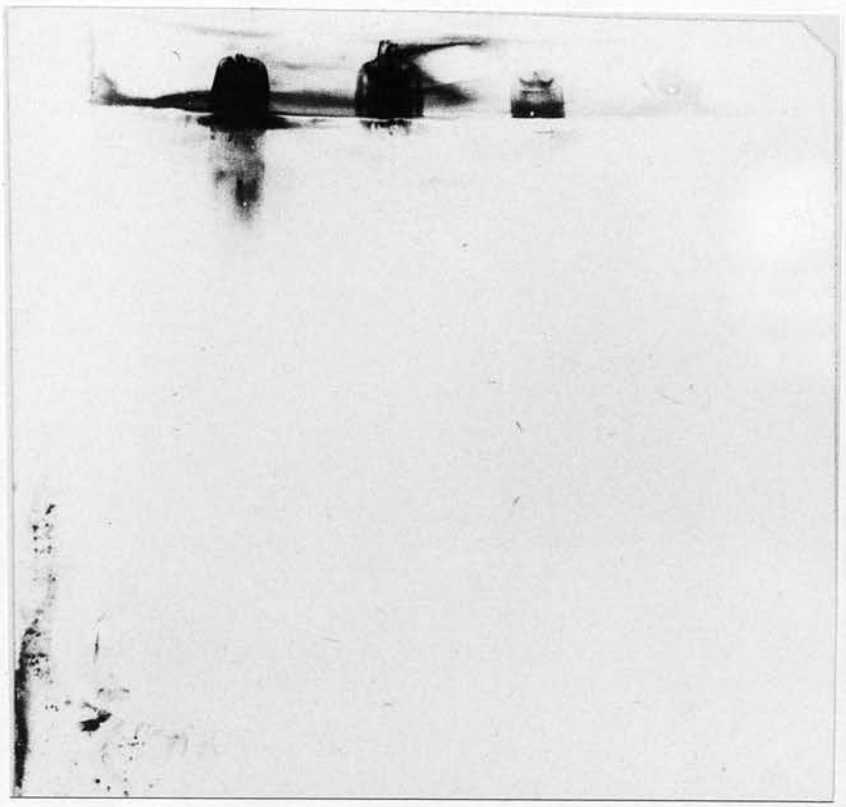


Figure 5.18 Immunoblot of MAb SZ 184 2.5.5 (O:18 specific) against LPS from *E. coli* 8b 281 (O:18) separated and blotted using the "PHAST" system. The loading of the tracks are a = 2µg; b = 0.4 µg, c = 0.2 µg. The arrow indicates the direction of electrophoresis.

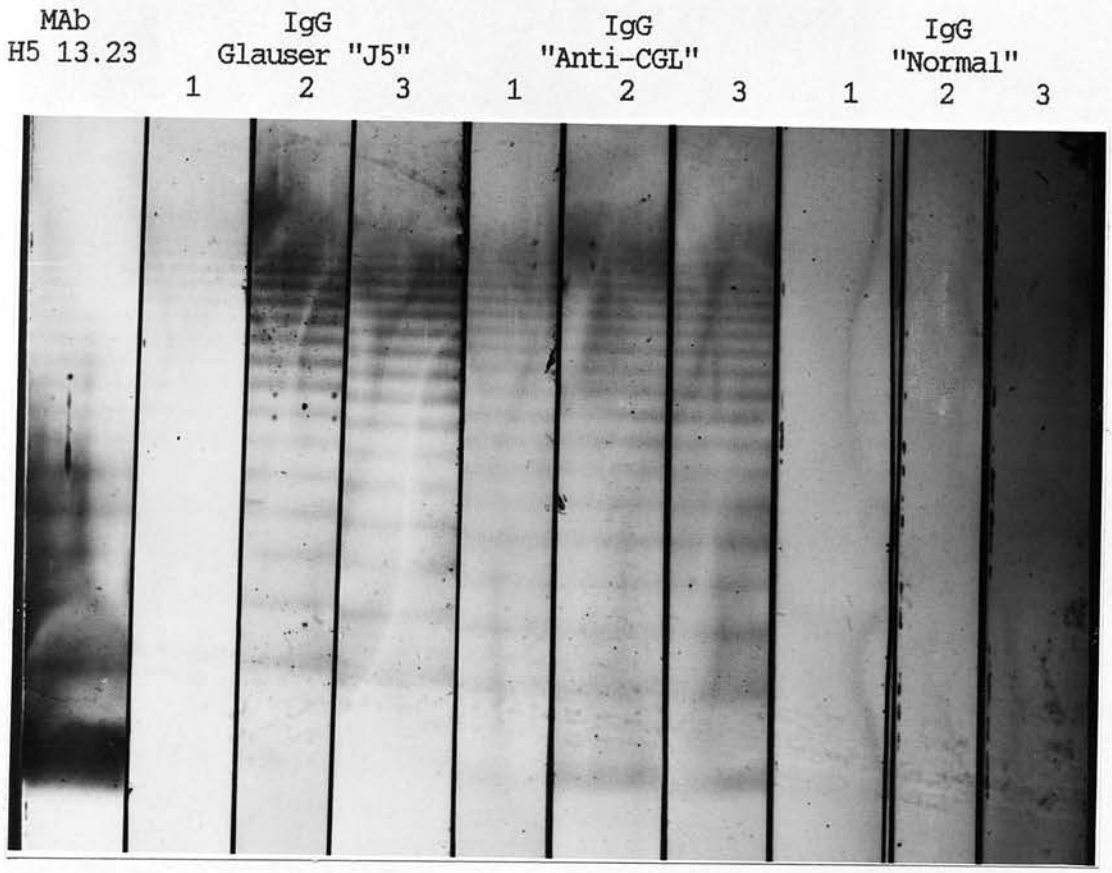


Figure 5.19 Immunoblot of human IgG preparations at three different concentrations, and MAb H5 13.23, against LPS (aqueous phenol preparation) from *E. coli* 8b 281 (O:18). Lanes were blotted separately with the antibodies shown above. Concentrations of human IgG preparations used were: 1 = 25 µg/ml; 2 = 250 µg/ml; 3 = 2500 µg/ml. MAb H5 13.23 was at a concentration of 19 µg/ml.

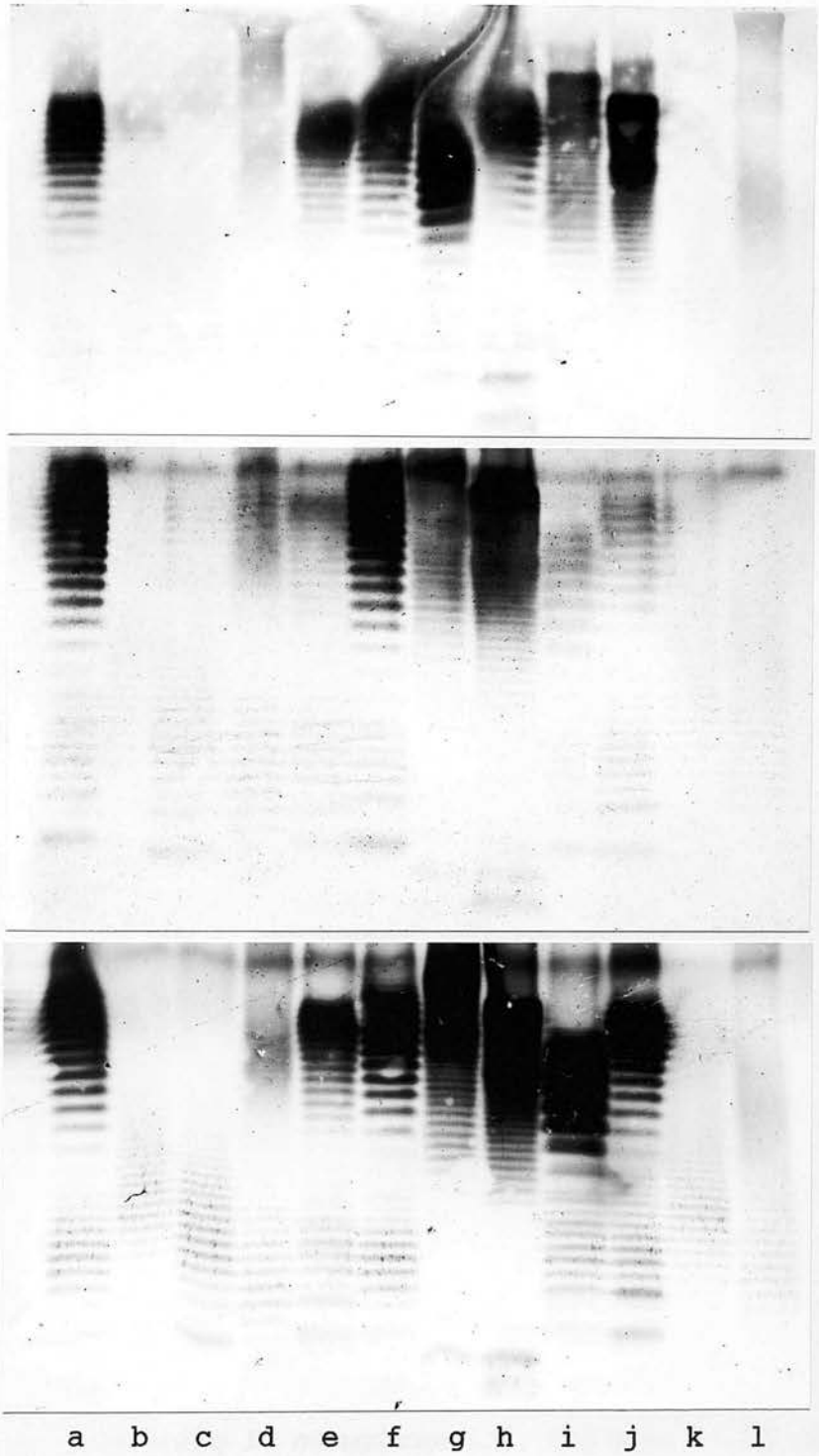


Figure 5.20 Immunoblot of human IgG preparations, (500µg/ml) against proteinase K digests of 12 blood culture isolates. Lane a = 8b226 (*E. coli*); b = 8b239 (*Proteus sp*); c = 8b257 (*Enterobacter cloacae*); d = 8b273 (*Serratia sp.*); e = 8b275 (*E. coli*); f = 8b281 (*E. coli*); g* = 8b300 (*Pseudomonas aeruginosa*); h* 8b305 (*Pseudomonas aeruginosa*); i* = 8b316 (*E. coli*) j* = 8b316 (*E. coli*); k = 9b24 (*Proteus sp.*); l = 9b26 (*Klebsiella pneumoniae*). *In the top print ("Normal" IgG) the two *Pseudomonas* strains are in lanes i and j , and *E. coli* strains 8b316 and 8b317 are in lanes g and h .

corresponding to the leading band of unsubstituted LPS core (as shown by the H5 13.23 control) while the "J5" preparation produced a barely visible band in this position.

The three IgG preparations were then reacted with the panel of 12 blood-culture isolates. All three gels were run simultaneously in order to produce comparable results. An IgG concentration of 500 µg/ml was used, which was between the two higher concentrations used above. The results are shown in figure 5.20. Note that in the blot of the "normal" IgG the order of lanes has been changed: *E. coli* strains 8b316 and 8b317 have been swapped with *P. aeruginosa* strains 8b300 and 8b305.

Binding of all three preparations to the higher part of the ladders of *E. coli* and *P. aeruginosa* is seen. For the "Normal" IgG and the "J5" IgG this is strongest with the two O:18 *E. coli* strains, while the "anti-CGL" IgG produced dense bands in this region with many of the strains. The leading bands are not strikingly different between the different IgG preparations, though it is difficult to locate them with confidence in the absence of a suitable control. Also visible is a set of narrow bands running ahead of the strongly-staining part of the O antigen ladders. These bands are seen in all lanes except those containing *P. aeruginosa* LPS, and seem to be identical in size and spacing in all lanes. These bands do not correspond to anything seen on the silver stains of the proteinase K preparations, and are thought to represent the enterobacterial common antigen.

DISCUSSION

The results presented here must be seen in their context as a small part of a much bigger project aimed at producing MAbs which could be used in therapy. I started from the point of trying to assess the binding of these MAbs to a wide range of clinically relevant bacteria. ELISA might have been the best approach, but at that time I did not have easy access to ELISA equipment within the department. After a brief attempt to examine the binding of MAbs to bacteria in ELISA I therefore concentrated on immunoblotting, which was available in the department, and briefly examined some other possible approaches. Plans were made for a more extensive ELISA-based investigation of MAbs and bacteria to be carried out later. When it became apparent that the MAbs shared the property of being limited in their specificity largely to *E. coli* I began to explore the possibility that some of them might be specific for particular core types of *E. coli*.

The ELISA results are limited, but did point to the major conclusion that, within the panel of organisms tested, most of the MAbs only recognised *E. coli* LPS. The antibodies tested were all known to react with purified LPS and therefore any reaction observed was likely to be with the LPS. The results suffer from the disadvantage that they were only performed at a single concentration of each MAb, and that they were not repeated. In the case of H7 41.76, the results appear to be wrong, in the sense that the broad cross-reactivity observed is incompatible with subsequent immunoblotting results reported in this chapter, and with ELISA results reported in chapter 6, and with the

results from other collaborators. This may result from some technical error in performing this ELISA assay, or from too high a concentration of MAb being used.

Immunoblotting is a cumbersome technique, not well suited to looking at the interaction of large numbers of bacteria and MAbs, but has produced some useful results. It has the advantage of showing graphically which component of a complex mixture is able to interact with the antibody, and in particular has been able to distinguish between binding to unsubstituted LPS core, substituted LPS core, and specific O antigen.

Immunoblotting was also able to indicate specificity of binding to particular core type of *E. coli* in a way that had not been suspected from previous ELISA data. This raised the possibility of using MAbs to determine the frequency of the known core types in a collection of wild-type *E. coli*, though the MAbs used in this chapter do not have the specificities required to do this. It is of interest to note at this point that the original reason for investigating the specificity of MAbs for different core types was the observation that MAbs bound different wild-type *E. coli* cores in a consistently different way, in particular the consistently poorer binding by 8b 317 of MAbs H4 1351.18 and H7 41.76. It was thought that this might be due to differential binding to different core types. Later results (chapter 6) found that all the wild-type *E. coli* strains used in these experiments were in the R1 core group, and therefore the differences observed here may be an artifact, or may indicate some heterogeneity within the R1 core group as subsequently defined with MAbs

Binding in immunoblots to a band corresponding to the interface between the stacking and separating gel was noted with some of the human IgG preparations (figure 5.20) and several MAbs (eg figures 5.5, 5.9, 5.10, 5.12). This band did not always occur, for example in the case of MAb H5 13.23, it is very strong in figure 5.15, but not in figure 5.6. When present tended to be present in all tracks, whether the antibody bound to LPS bands in the track or not. This band is thought to represent non-specific binding to proteinase K or to aggregates of LPS. It was clearly distinct from the diffuse slow-moving material seen in some of the silver-stains and blots of rough LPS. This latter material only reacted with antibody when reaction with the leading band was seen, and therefore can be interpreted as specific binding to non-covalent polymers of rough LPS [184].

The binding of SZ 30 4.2.8 to the slow-moving bands of O:18 LPS in addition to its binding to the fast-moving band of the other strains tested implies some cross-reactivity between the O-specific polysaccharide and core or lipid A. SZ 30 4.2.8 was a cross-reactive MAb which resulted from of an immunisation with lipid A and therefore was expected to react with lipid A. The only obvious similarity between the structure of lipid A and the O:18 antigen [90] is the presence of two glucosamine residues. These residues are however N-acetylated in the O:18 antigen, unlike those in lipid A. One of the glucosamine residues in the O:18 antigen is ß-1 linked to another sugar and may form an epitope similar to similar to that described by Kuhn et al [122] as specificity "f" on lipid A.

None of the other methods used here proved useful in examining the binding of MAbs to a LPS from a wide range of species. Co-agglutination and PHAST system gels failed on simple technical grounds since the co-agglutination reagent was auto-agglutinated, and LPS failed to enter the PHAST system gel. Flow cytometry was rejected for more complex and important reasons, however. The failure of the MAbs tested to bind whole bacteria when they have been shown to bind LPS from those bacteria clearly indicates that the appropriate epitopes are not exposed on these bacteria. This is relevant to therapy with such antibodies as well as to *in vitro* studies. The situation in the infected host may however not be reflected in these experiments. This question has been studied further by Nelson et al [165,166] who have shown that the conditions of bacterial growth markedly affect the binding of MAbs to live bacteria.

The results obtained with human IgG preparations underline the non-quantitative nature of immunoblotting. Major differences can be demonstrated in the amount of anti-LPS-core antibody in these preparations (GR Barclay: personal communication) but these are not clearly represented in the immunoblot.

The immunoblot with human IgG preparations also demonstrated the presence in the proteinase K preparations of a set of bands which were not detected in the silver stain. Their survival after proteinase treatment suggests that they are carbohydrate in nature, and their presence and identical size in all of the enterobacterial preparations together with their absence from the *P. aeruginosa*

preparations suggests that they represent the enterobacterial common antigen (ECA) [121]. The failure to visualise ECA in the silver stain and nature of the bands seen in the immunoblot is consistent with the results reported by Peters et al [183].

Overall, the work described in this chapter gave me practical experience of some of the methods available to study cross-reactive antibodies to LPS, and led to the application of some of these antibodies to the characterisation of core types in clinical isolates of *E. coli*, as described in the next chapter.

CHAPTER 6

FREQUENCIES OF LPS-CORE TYPES AMONG CLINICAL ISOLATES OF *E. COLI* DEFINED WITH MONOCLONAL ANTIBODIES

In wild-type smooth *E. coli* the core region can occur in at least 4 different forms, known as R1, R2, R3, and R4 [207-209]. The naturally rough strain K12 has another different core region [102]. These core types were defined by a combination of phage-typing and serological methods, and their chemical structure subsequently determined [110]. The O antigen of smooth LPS may mask some of the epitopes and phage attachment sites in the core region, and may contain some of the same carbohydrate residues as the core region. The serological phage-typing and chemical methods which were used to define the different core types are therefore not directly applicable to wild-type smooth strains. The frequency with which these different core types occur in wild-type *E. coli* is therefore not known.

Little is known about the association between core types and O-serotypes of *E. coli*, except that the original R1 and R2 rough mutants were both derived from O8 strains [209], and that the original R3 mutant was derived from an O111 strain [207]. There was no published evidence at the time this work was done on the relationship of LPS-core types with capsular types, or with virulence. Information about the distribution of O and K antigens has proved to be useful, for example in identifying and understanding enteropathogenic strains [178], and in defining the range of antibodies for O- or K-specific antisera for treatment of bacteraemia [52,53]. Information about core types might be equally interesting. We therefore set out to select, from the available MAbs, a panel

which would react with specific LPS-core types of *E. coli*. We wished to use these MAbs as a means of determining the LPS-core types of a collection of wild-type *E. coli*.

It was decided to use heat-killed cells were used as antigen, since it had been found by others [41,164] that the binding of core-specific MAbs in ELISA was increased when heat-killed cells were used instead of unheated cells. Only a single dilution of MAb could be used, since the experiment would otherwise have become unmanageably large.

MAbs

A panel of 61 MAbs was selected from a total pool of 300 produced jointly in Basel and Edinburgh. The selection was based on the results of LPS-polymyxin ELISA against the panel of purified LPS antigens listed in table 2.1. The data from these studies was sorted by the correlation method devised by Dr GR Barclay (described below) to identify MAbs with similar profiles of reactivity with the panel of LPS. Where several MAbs had very similar profiles, only a single representative was chosen. Concentrations were not known for every MAb preparation, but were in the range of 10-50 µg/mL.

Two batches of MAb were used. Batch 1 MAbs were used in the first large ELISA experiment with the blood-culture isolates. Batch 2 MAbs were used for the immunoblots and for experiments with the urine and faecal strains.

Bacteria and Bacteriophage

E. coli R1 (F470), R2 (F576), R3 (F653), R4 (2513) and K12 (2131) were provided by Dr H. Brade, Forschungsinstitut, Borstel, Germany. These had been distributed in 1990 to all the laboratories collaborating with Sandoz because of some discrepancies between the strains previously used in the different laboratories. In addition the *E. coli* R1, R2, R4 and K12 strains from the MPRL collection (listed in table 5.1) were used.

E. coli O:1, O:2, O:4, O:6, O:8, O:12, O:15, O:16, O:18, O:75 and O:86 were obtained from Dr A S Cross, Walter Reed Army Institute, Washington. Five strains of *E. coli* O:18 were obtained from this source: O:18 K1, its isogenic mutants O:18K1$^-$(non-capsulate) and O:18 Krf (rough mutant); O:18 K5, and its non-capsulate mutant O:18K5$^-$.

Rough-specific phages FO, Br10, C21, 6SR and T4 [208,209], and their bacterial hosts were obtained from Dr. G Schmidt, Forschungsinstitut, Borstel, Germany. Phage specific for *E. coli* capsular types K1 (øK1GS) and K5 (øK5DG) were obtained from Dr AP Roberts, Charing Cross and Westminster Medical School, London, UK.

Blood-culture isolates and urine isolates were obtained from the Clinical Bacteriology Laboratory, Department of Medical Microbiology, Edinburgh University, Edinburgh, UK, from routine clinical specimens. The blood-culture isolates were all the Gram-negative organisms isolated in a one-year period. Twenty-one faecal isolates of *E. coli* were obtained, each from a separate healthy volunteer.

Rough mutants of *Salmonella minnesota* (R60), *Salmonella typhimurium* (1542), *Klebsiella pneumoniae* (M10B), and *Pseudomonas aeruginosa* (PAC608), and wild-type smooth *Shigella sonnei* (colicin types 4/2 and 2/1) and *Shigella flexneri* (serotypes 1a and 3) were freeze-dried stock cultures held in the laboratory. *Shigella dysenteriae* and enteropathogenic *E. coli* were stock cultures or clinical isolates held on agar slopes. Three isolates of *E. coli* O:157 were provided by Dr M Hanson, Central Microbiological Laboratories, Western General Hospital, Edinburgh. The O type of all *Shigella* and enteropathogenic

E. coli strains was confirmed by slide agglutination with appropriate antisera before use.

Heat-killed bacterial ELISA used with monoclonal antibodies

Overnight nutrient broth cultures of bacteria were washed and resuspended in saline to give an E_{525} of 0.5, and heated for 30 min at 100°C. Cells were then diluted 1 in 20 in coating buffer (sodium carbonate/bicarbonate pH 9.6 with 0.05% sodium azide) and 100 µl added per well to NUNC (Roskilde, Denmark) polysorb strips. After overnight incubation at room temperature plates were washed three times with wash buffer (0.05% Tween 20 and 0.05% sodium azide in PBS pH 7.4) and post-coated with 5% wt/vol bovine serum albumin in coating buffer overnight at room temperature. Plates were then washed and stored at -20°C.

MAbs were diluted 1 in 250 in dilution buffer (4% wt/vol polyethylene glycol, 0.05% sodium azide, and 0.05% Tween 20 in PBS pH 7.4). Diluted MAb (100 µl) was added to wells and incubated for 90 min at 37°C. Plates were washed three times and urease-conjugated anti-mouse Ig (Zymed, Cambridge, UK; diluted 1 in 500 in dilution buffer with 0.5% bovine serum albumin, 100 µl per well) was added and incubated for a further 90 min. Plates were then washed three times in wash buffer and three times in distilled water, urease substrate solution (Seralab, Crawley Down, Sussex, UK; 100 µl per well) added, incubated for 90 min at room temperature, and read at 590 nm. Blank readings from wells which contained no antigen was subtracted.

Serum sensitivity of bacteria

Serum from one donor (APG) was stored at -70°C. This serum had IgG antibodies to LPS core at a level equal to the 65th centile of the normal population [211]. Overnight broth cultures were washed and resuspended in Complement Fixation Test (CFT) buffer (Oxoid, Basingstoke, UK) at room temperature to an E_{525} of 0.5. This suspension was then diluted 1 in 5000 in CFT buffer, and 10 µl added to 70 µl CFT buffer and 20 µl freshly thawed serum or heated (56°C, 30 min) serum. After 1 h at 37°C duplicate 20 µl samples were spread on the surface of nutrient agar plates and colonies counted after overnight incubation. If the number of cfu in the fresh serum was less than 10% of the number in the heated serum the organism was considered to be serum sensitive.

O typing

E. coli strains from blood and urine cultures were screened for common O antigens, by ELISA, with absorbed O typing sera (Difco, Detroit, Michigan) to O:1, O:2, O:4, O:6, O:7, O:12, O:15, O:18, O:22 and O:75 (diluted 1 in 1000), and by tube agglutination at a final dilution of 1 in 80 with unabsorbed sera to O:8 and O:9 (Statens Seruminstitut, Copenhagen, Denmark). O types were confirmed by conventional tube agglutination [177]. This method is discussed in more detail in the Note at the end of this chapter.

Phage typing

Overnight broth culture was inoculated as a lawn on nutrient agar and allowed to dry. Phage suspension (10 µl) was dropped onto the agar and incubated overnight. A clear zone of lysis was scored as sensitive.

Correlation sorting of ELISA data

This method for analysing data from ELISA experiments involving very large numbers of antibodies and antigens was devised and implemented by Dr GR Barclay.

For any pair of antibodies which have both been reacted in ELISA with an identical large panel of antigens the pairs of OD results for each antigen can be used to calculate Pearson's correlation coefficient as a measure of the similarity between the overall pattern of results. A similar calculation can be made for any pair of antigens which have both been reacted with reacted with an identical large panel of antibodies.

A programme was written by Dr Barclay which calculated the correlation coefficient for each possible antibody pair, and for each possible antigen pair. The antibodies and antigens were then rearranged by the programme so that pairs with high correlation coefficients were placed together. The result of this manipulation is a table in which similar antibodies and antigens are grouped together.

Reaction of batch 1 MAbs with blood culture isolates of *E. coli*

A single large ELISA was designed involving the 61 MAbs and 111 bacteria, including 80 blood culture isolates said to be *E. coli*, and 28 standard strains. (Also included were three isolates from sheep with watery mouth: these will be ignored). This had two aims: It would give an indication of the degree of cross-reactivity of the MAbs with the strains being studied, and, in addition, since some of the MAbs were known to be selective for particular core types, it was hoped to identify the core type of some or all of the wild-type strains.

The sorted results obtained with the blood-culture isolates of *E. coli* and controls are shown in figure 6.1. MAbs which reacted with a restricted range of R types and also with some smooth strains of *E. coli* were identified from these results in order to determine the core type of wild-type smooth strains. Four such distinct patterns of reactivity were observed, each in a group of MAbs which were placed together by the correlation-sorting process.

a)"R1-specific" MAbs (SZ 43 3.4.8, WN2 423.10, and SZ 43 27.11.2) reacted with R1 and with *E. coli* O:1, O:2, O:4, O:6, O:8, O:16, O:18,and O:75, and with many of the wild-type strains.
b)"R2-specific" MAbs (H4 361.23, F6 724.02) reacted with R2 and O:12, and with a different group of wild-type strains.
c)"R3-specific" MAbs (H4 250.7, W4 39.11, H4 351.05b and W4 434.07)

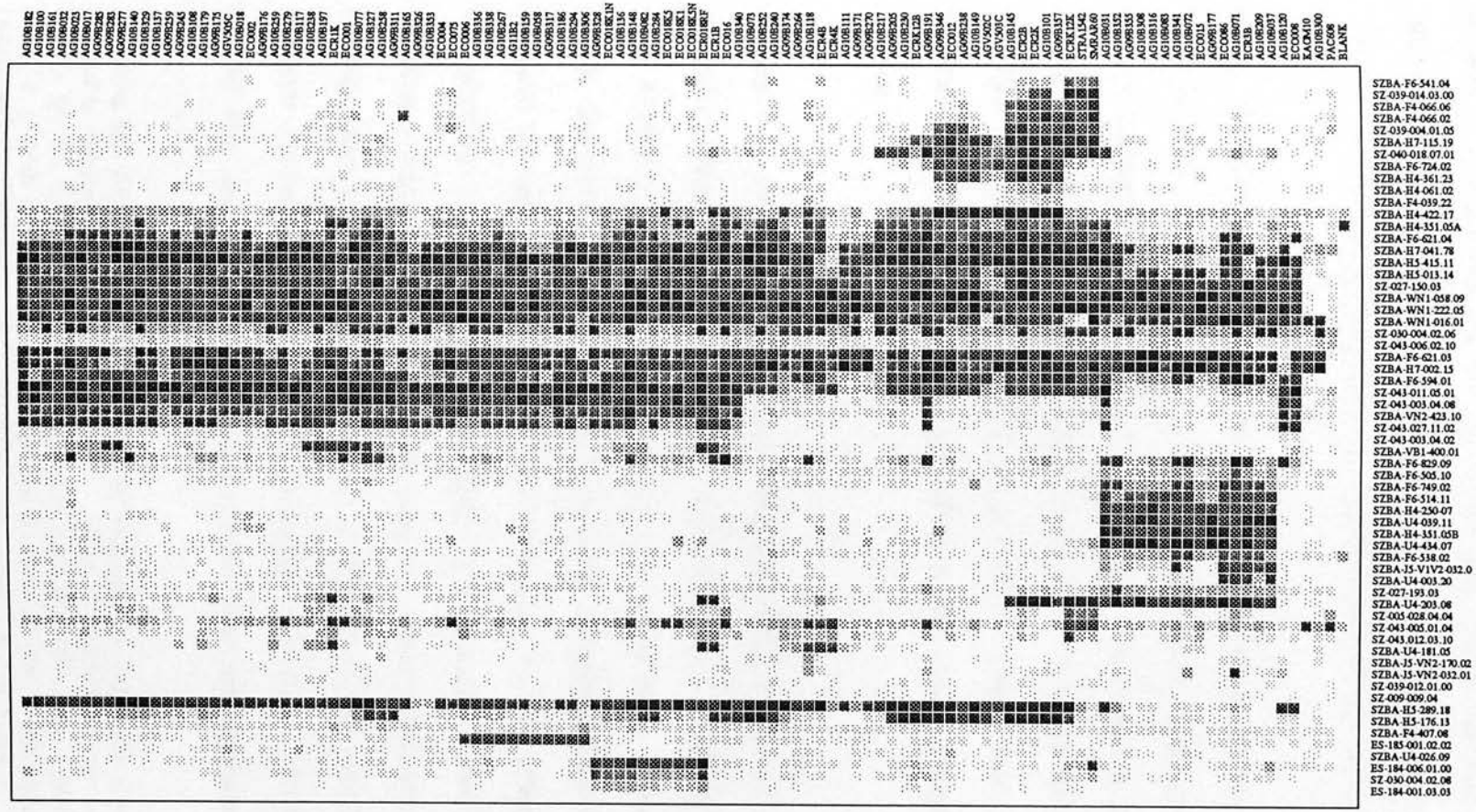


Figure 6.1 Correlation-sorted ELISA OD results for 61 MAbs and 112 heat-killed bacteria. Bacteria are coded as follows: **AG** = blood-culture isolate; **AGV** = strain from sheep with Watery Mouth; **ST** = *Salmonella typhimurium*; **SM** = *Salmonella minnesota*; **KA** = *Klebsiella pneumoniae*; **PA** = *Pseudomonas aeruginosa*. **EC** = standard strains of *E. coli* with O, R and K type as indicated. Suffix N = non-capsulate mutant. Suffix B on the *E. coli* rough strains indicates that the strain is from Borstel. Other rough strains of *E. coli* have the suffix K. OD indicated by shading from □ = 0 to ■ = 2.0

reacted with R3, O:15 and O:86, and with a third group of wild-type strains.

d)"all *E. coli*" MAb SZ 27 150.3 and WN1 222.5 reacted with all the *E. coli* rough and smooth strains[1] and with the *Salmonella* rough mutants but not with the *Klebsiella* or *Pseudomonas* rough mutants.

Other patterns were observed, including a group of MAbs which reacted with R2 and K12 or with R2 and the *Salmonella* rough mutants, or with all of these strains. However no MAb was found which reacted with K12 or R4 but not with other rough mutants. Neither MAb SZ 27 150.3 nor WN1 222.05 nor any of the core-specific MAbs mentioned above reacted with the *Klebsiella* or *Pseudomonas* rough mutants which were tested.

Figure 6.2 shows the OD values obtained with three of the core-type-specific MAbs against the 79 *E. coli* strains. Most of the strains show strong reactivity with one of the three MAbs. The order in which the strains are presented is that determined by correlation-sorting of the data, and this has resulted in the ordering of most of the strains into four groups. The first group consisted of 47 strains (10b182 - 10b340) which reacted almost exclusively with the R1 MAb, and these were classed as R1 strains. Two smaller groups of 6 strains (9b346 - 9b357) and 11 strains (10b352 - 10b037) react with the R2 MAb and the R3 MAb respectively, and are classed as R2 and R3.

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1. MAbs SZ 27 150.3 and WN1 222.5 failed to bind to one of the blood-culture isolates, strain 10b300. This strain was re-identified and found to be a *Citrobacter*. 10b300 was one of two isolates from a single blood culture. The second of these two isolates, 10b301, was recorded as being a *Citrobacter*, but was in fact an *E. coli*. It was concluded that two isolates had been interchanged.

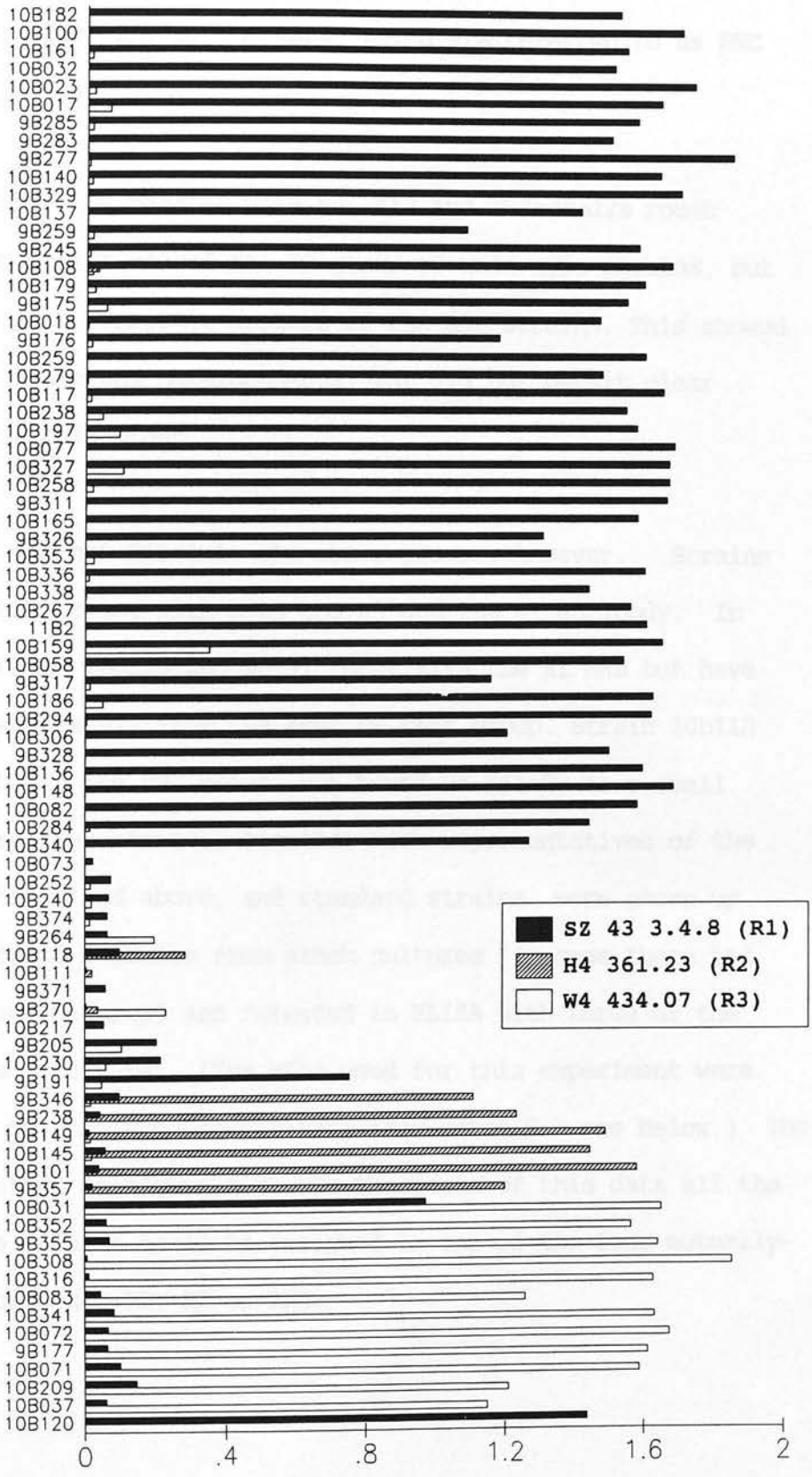


Figure 6.2 Binding (ELISA OD) of three core-type-specific MAbs (batch 1) to 79 blood-culture isolates of *E. coli*.

The fourth group consists of 12 strains (10b73 - 10b230) which do not react strongly with any of the MAbs, which are referred to as RNC (not classified).

Those MAbs which had reacted with R2, K12 and *Salmonella* rough mutants reacted with all of the R2 group of wild-type strains, but also reacted with variable numbers of the RNC strains. This showed that the RNC group was heterogeneous, but did not permit clear subdivision of this group.

Some strains did not fit into the above groups, however. Strains 10b31 and 10b159 react with both the R3 and the R1 antibody. In addition, strains 10b120 and 9b191 react with the R1 MAb but have been sorted separately from the rest of that group. Strain 10b118 appeared to be in the RNC group, but bound H4 361.23 to a small extent. These five strains, together with representatives of the four groups described above, and standard strains were grown up again from single colonies from stock cultures (in case there had been any mixed cultures) and retested in ELISA with three of the core-type-specific MAbs. (The MAbs used for this experiment were preparations of confirmed specificity from batch 2 - see below.) The results are shown in figure 6.3. On the basis of this data all the blood-culture strains could be assigned to one of the four mutually-exclusive groups R1, R2, R3 or RNC.

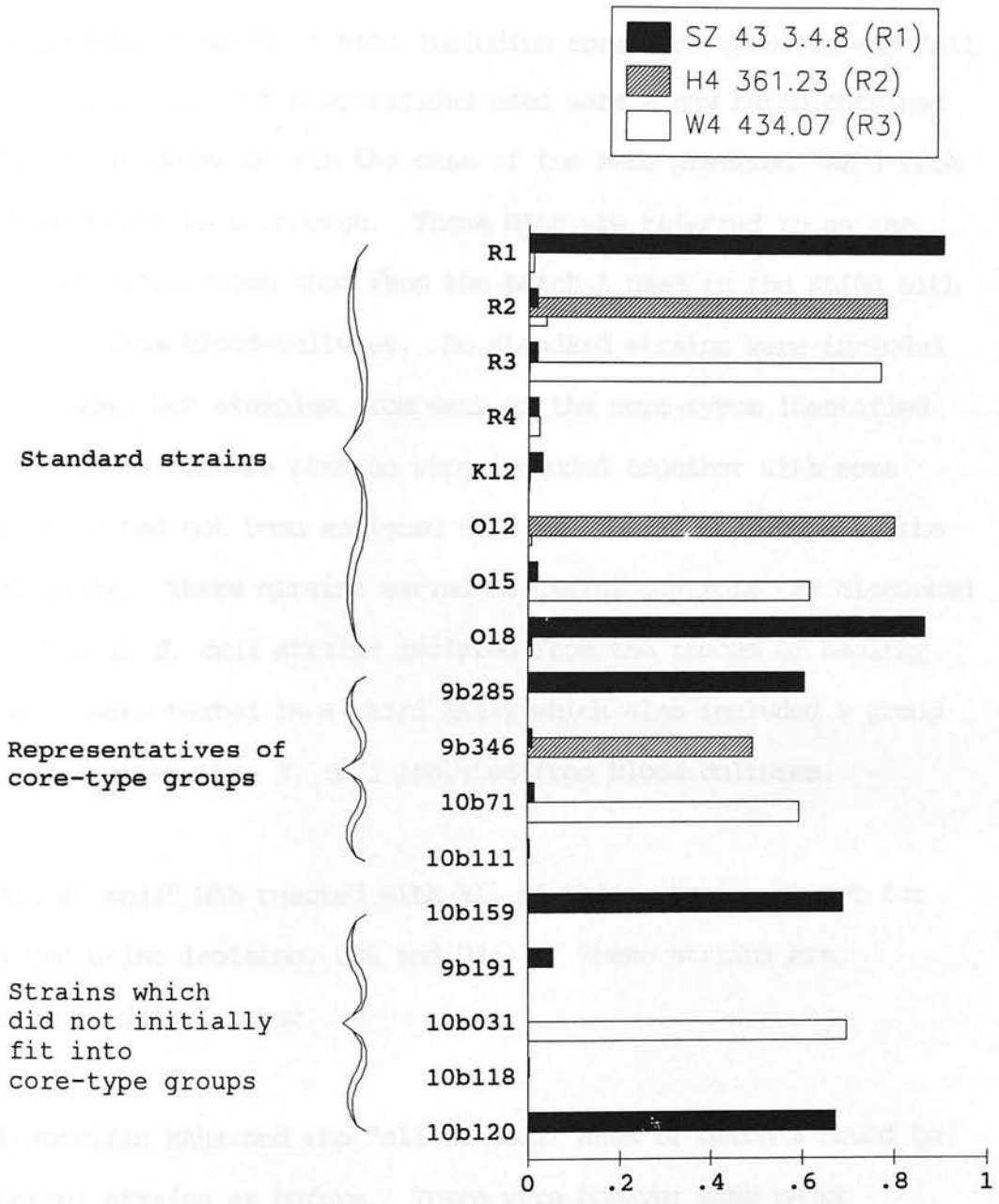


Figure 6.3 Binding (ELISA OD) of three core-type-specific MAbs (batch 2) to selected *E. coli* strains.

Urine and Faecal strains of *E. coli*: Batch 2 MAbs

The collection of 80 *E. coli* strains from urine cultures was tested in a ELISA with a panel of MAbs including core-type-specific and "all *E. coli*" MAbs. The MAb preparations used were a new batch obtained from Sandoz in Basel or (in the case of the MAbs prefixed "SZ") from local production in Edinburgh. These MAbs are referred to as the batch 2, to distinguish them from the batch 1 used in the ELISA with the *E. coli* from blood-cultures. No standard strains were included in this assay, but examples from each of the core-types identified among the blood culture strains were included together with some strains that had not been assigned to a particular core type by the initial assay. These strains served as useful controls, as discussed below. The 21 *E. coli* strains isolated from the faeces of healthy volunteers were tested in a third assay which also included a group of bacteria other than *E. coli* isolated from blood cultures.

The "all *E. coli*" MAb reacted with all of these strains except for two of the urine isolates, U36 and U44. These strains are discussed in detail later.

The R1-specific MAbs and the "all *E. coli*" MAbs of batch 2 bound to the control strains as before. There were however some major differences noted in the reactions of some of the R2 and R3 core-type-specific MAbs of batch 2 with control strains. This seemed to relate particularly to MAbs which had been received from Basel, and could be explained by misidentification of some MAbs.

R2-specific MAbs in batch 2

H4 361.23 (R2 specific in batch 1) bound to four of the faecal isolates, none of which reacted with the R1 antibody. These four strains were identified provisionally as R2 strains. MAb H4 361.23 was however of very low activity in the assay of the urine strains. MAb F6 724.02 (also R2 specific in batch 1) bound to two faecal strains which were neither in the R1 group nor in the provisional R2 group of faecal strains. It also bound to four of the urine strains,and to the control strains of R3 core-type. It appeared therefore that batch 2 of MAb F6 724.02 was R3 specific.

The MAb of batch 2 which appeared to react most clearly with the R2 control strains tested was H4 422.17. This MAb also reacted strongly with the four provisionally identified R2 strains among the faecal isolates and with four of the urine strains. Batch 1 of MAb H4 422.17 had reacted most strongly with the R2 strains in the original assay of *E. coli* from blood cultures but had also reacted weakly with some other strains. The difference between the two batches might be explained by differences in antibody concentration. Results obtained with H4 422.17 (batch 2) were used to identify provisionally a group of R2 strains among the strains from urine.

R3-specific MAbs in batch 2

MAb H4 250.7 (R3 specific batch 1) was found to be widely cross-reactive with organisms other than *E. coli*, and is discussed further in chapter 7. H4 351.05b (R3 specific in batch 1) reacted with the

R2 control strains, and W4 39.11 (R3 specific in batch 1) was no longer available. W4 434.07 (R3 specific in batch 1) did react with the R3 control strains as expected, and also reacted with four urine strains and two faecal strains which were not in the R1 group nor in the provisional R2 group. The activity of MAb W4 434.07 of batch 2 was therefore consistent with its activity in batch one, and it was used to identify the R3 group of urine and faecal strains.

Confirmation of the specificity of batch 2 MAbs

A sufficient volume of seven of the MAbs from batch 2 discussed above was available for more detailed testing. Repeat testing of these preparations with the control organisms confirmed that the correct conclusions had been drawn above about the specificity of these MAbs. (table 6.1). MAbs F6 724.02 and H4 250.7 of batch 2 clearly had different specificities from the batch 1 MAbs with the same reference number. Batch 2 did however contain one R3-specific MAb (W4 434.07) one R2-specific MAb (H4 361.23) and three R1 specific MAbs (SZ 43 3.4.8, SZ 43 27.11.2 and VN2 423.10) which had the specificities expected from batch one. This latter group of five MAbs were used in further experiments.

Confirmation of the classification of the *E. coli* strains from faeces and urine

The faecal strains (with the possible exception of strain F1) could be easily be assigned to mutually exclusive groups of strains, based on their reactions with batch 2 MAbs SZ 43 3.4.8 (R1 specific) H4

MAb No.	Reactivity (ELISA OD) with *E. coli* strains:								Specificity	
	R1	R2	R3	R4	K12	O12	O15	O18	Batch 1	Batch 2
SZ 43 3.4.8	0.904	0.018	0.018	0.021	0.031	0.022	0.020	0.865	R1	R1
SZ 43 27.11.2	0.310	0.012	0	0.014	0.012	0.032	0.011	0.406	R1	R1
VN2 423.10	0.605	0.002	0	0.006	0.003	0.008	0.092	0.647	R1	R1
H4 361.23	0	0.781	0.001	0	0.003	0.799	0.002	0.004	R2	R2
F6 724.02	0	0	1.019	0.005	0.012	0.028	0.956	0.012	R2	R3
W4 434.07	0.011	0.038	0.768	0.024	0.001	0.007	0.614	0.008	R3	R3
H4 250.7	0.145	0.470	0.295	0.195	0.270	0.904	0.212	0.270	R3	Cross

Table 6.1. Reactivity (ELISA OD) of MAbs from batch 2 with laboratory strains of *E. coli*.

361.23 (R2 specific) and W4 434.07 (R3 specific) (figure 6.4). The other R1-specific MAbs (VN2 423.10 and SZ 43 27.11.2), and the other batch 2 MAbs identified as R2 or R3 specific gave consistent results. The validity of these conclusions was confirmed in a repeat experiment retesting the binding of these three MAbs to examples from each group together with the rough mutants R1, R2, and R3, and with the smooth strains O:12 O:15 and O:18 (figure 6.5). Strain F1, which had reacted weakly with the R1-specific MAbs, was included in this assay and subsequently assigned to the RNC group.

Most of the urine strains could also be assigned to R1, R2, R3 or RNC groups based on their reactions with SZ 43 3.4.8 (R1 specific), H4 422.17 (provisionally R2 specific) and W4 434.07 (R3 specific), as shown in figure 6.6. As with the blood-culture strains, strains which did not fall clearly into one group were re-tested. In addition, all the strains provisionally identified as belonging to the R2 group were re-tested with the confirmed R2-specific batch 2 MAb H4 361.23. As shown in figure 6.7, the group of strains provisionally identified as R2 did react with H4 361.23, and the other strains could all be placed in one of the four mutually-exclusive groups R1, R2, R3 or RNC.

Urinary strains which did not react with the "all-*E. coli*" MAbs

Strains U36 and U44, the urine isolates which had not reacted with the "all *E. coli*" MAbs, were retested in ELISA, and the lack of reactivity with these MAbs under the standard conditions used was confirmed. The identification of U36 was checked by API 20E, and

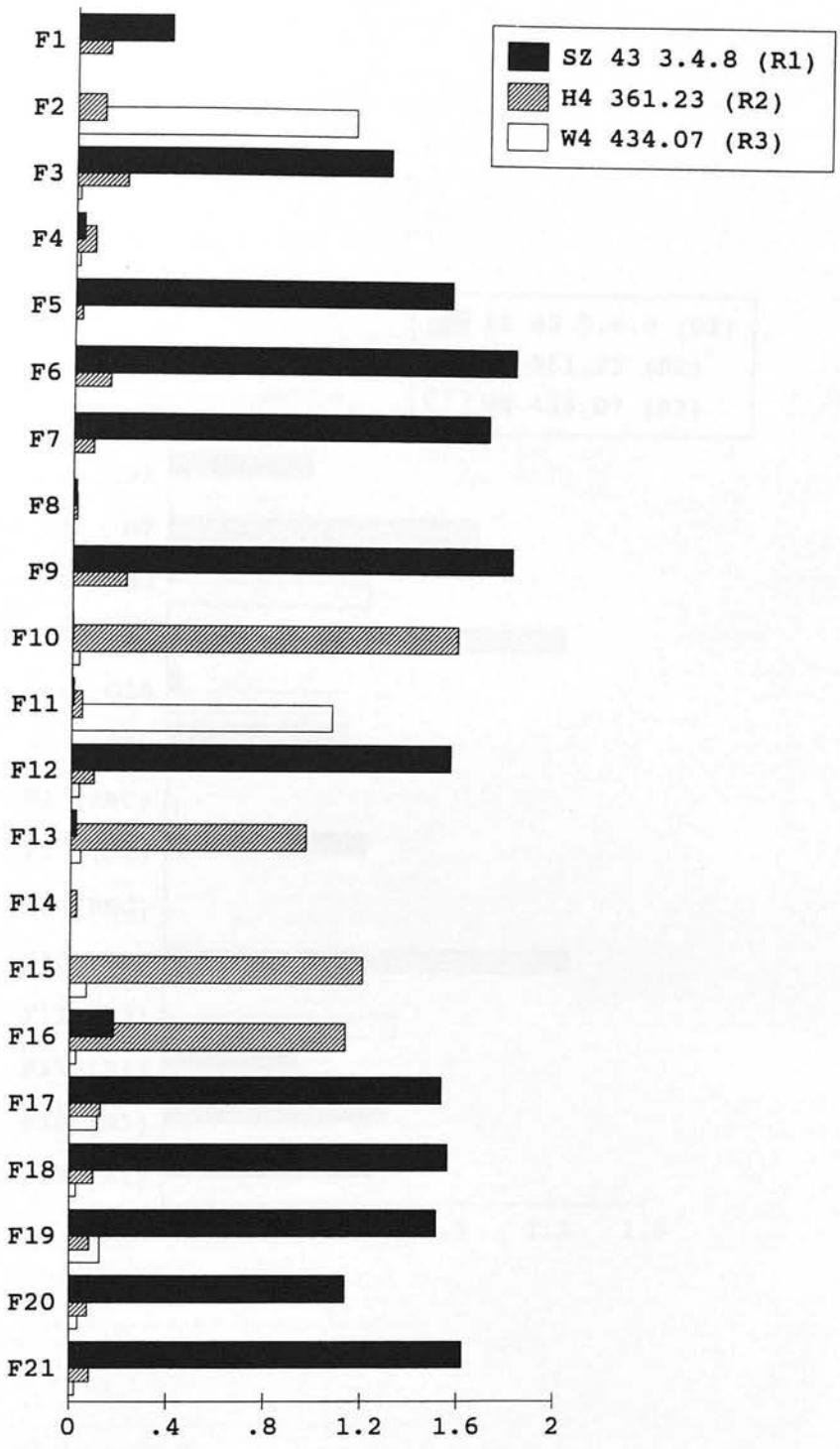


Figure 6.4 Binding (ELISA OD) of three core-specific MAbs (batch 2) to 21 *E. coli* strains from the faeces of healthy volunteers.

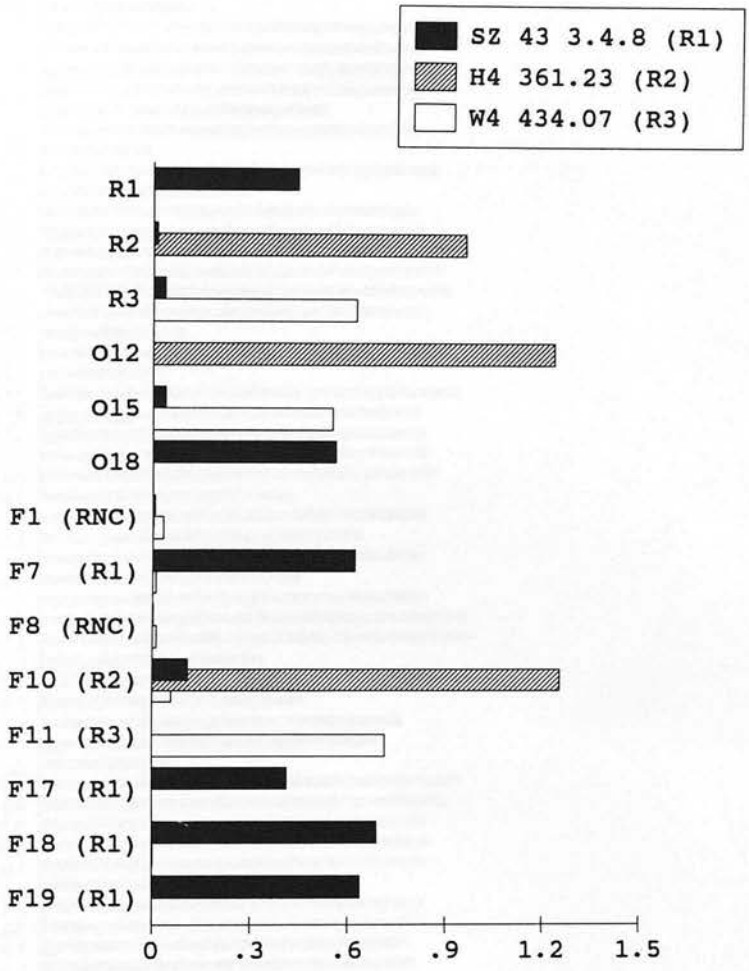


Figure 6.5 Repeat assay of binding (ELISA OD) of core-specific MAbs to selected faecal *E. coli* strains and controls.

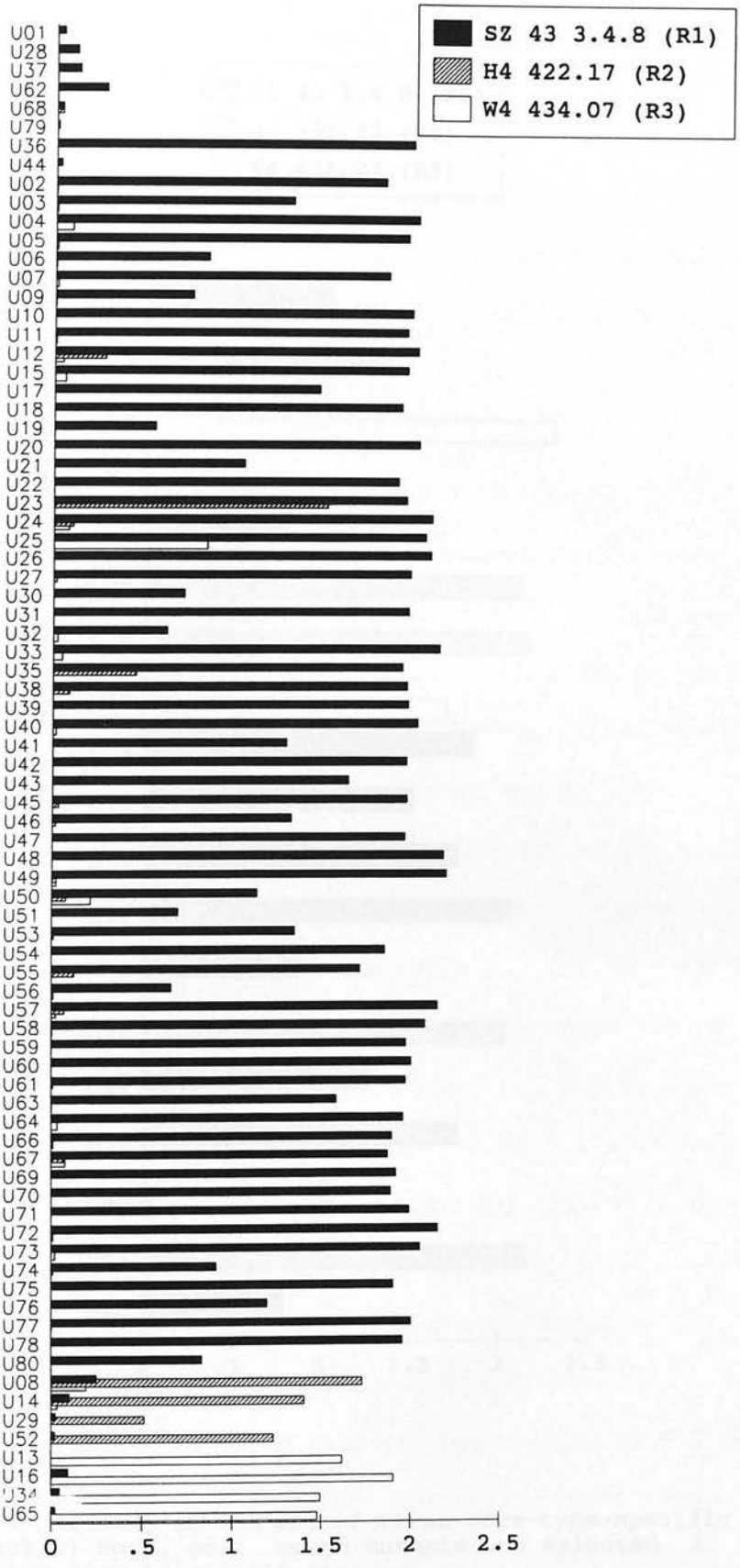


Figure 6.6 Binding (ELISA OD) of three core-type-specific MAbs (batch 2) to 80 *E. coli* strains from urine cultures.

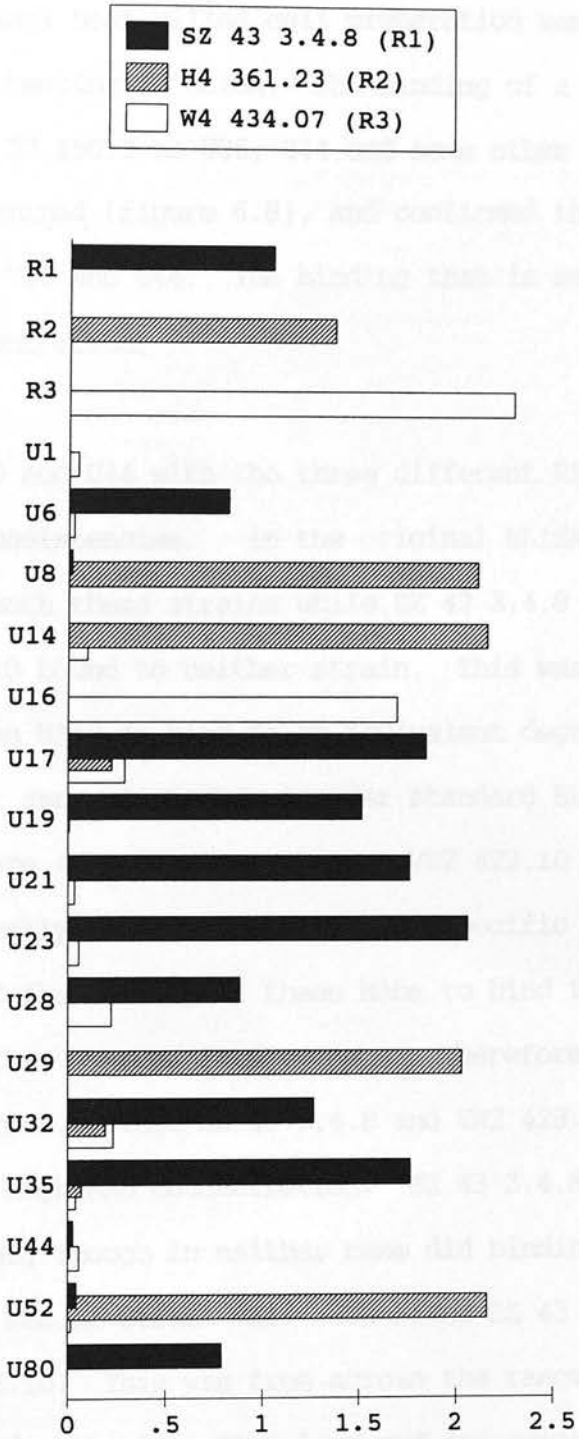


Figure 6.7 Binding (ELISA OD) of three core-type-specific MAbs (batch 2) to *E. coli* rough mutants and selected *E. coli* strains from urine cultures.

confirmed to be *E. coli*. Strain U44 was unfortunately no longer viable, but the original heat-killed cell preparation was however available for repeat testing in ELISA. The binding of a range of concentrations of SZ 27 150.3 to U36, U44 and some other *E. coli* from urine culture was examined (figure 6.8), and confirmed that there was much less binding to U36 and U44. The binding that is seen is at much higher MAb concentration.

The reactivity of U36 and U44 with the three different R1-specific MAbs showed some inconsistencies. In the original ELISA screen, SZ 43 27.11.2 bound to both these strains while SZ 43 3.4.8 bound only to U36, and VN2 423.10 bound to neither strain. This was in contrast to the trend for these MAbs to bind to an equivalent degree to "R1" strains. In a repeat assay (not shown) under standard ELISA conditions results were as above, except that VN2 423.10 now bound to U36, although more weakly than the other two R1-specific MAbs. A quantitative assay of the ability of these MAbs to bind to these two organisms and some other *E. coli* from urine was therefore performed. This confirmed (figure 6.9) that SZ 43 3.4.8 and VN2 423.10 did not bind to U44, even at high MAb concentration. SZ 43 3.4.8 and VN2 423.10 did bind to U36, though in neither case did binding to U36 equal the binding to the R1 strain U21. U36 bound SZ 43 3.4.8 more than it bound VN2 423.10. This was true across the range of concentrations, both for absolute OD values and for comparison with binding to the R1 strain. SZ 43 27.11.2 is shown in figure 6.9 to be less specific in its binding, producing, at higher concentrations, measurable OD values with all the *E. coli* strains tested in this assay. This is consistent with other work with SZ 43 27.11.2 [164]

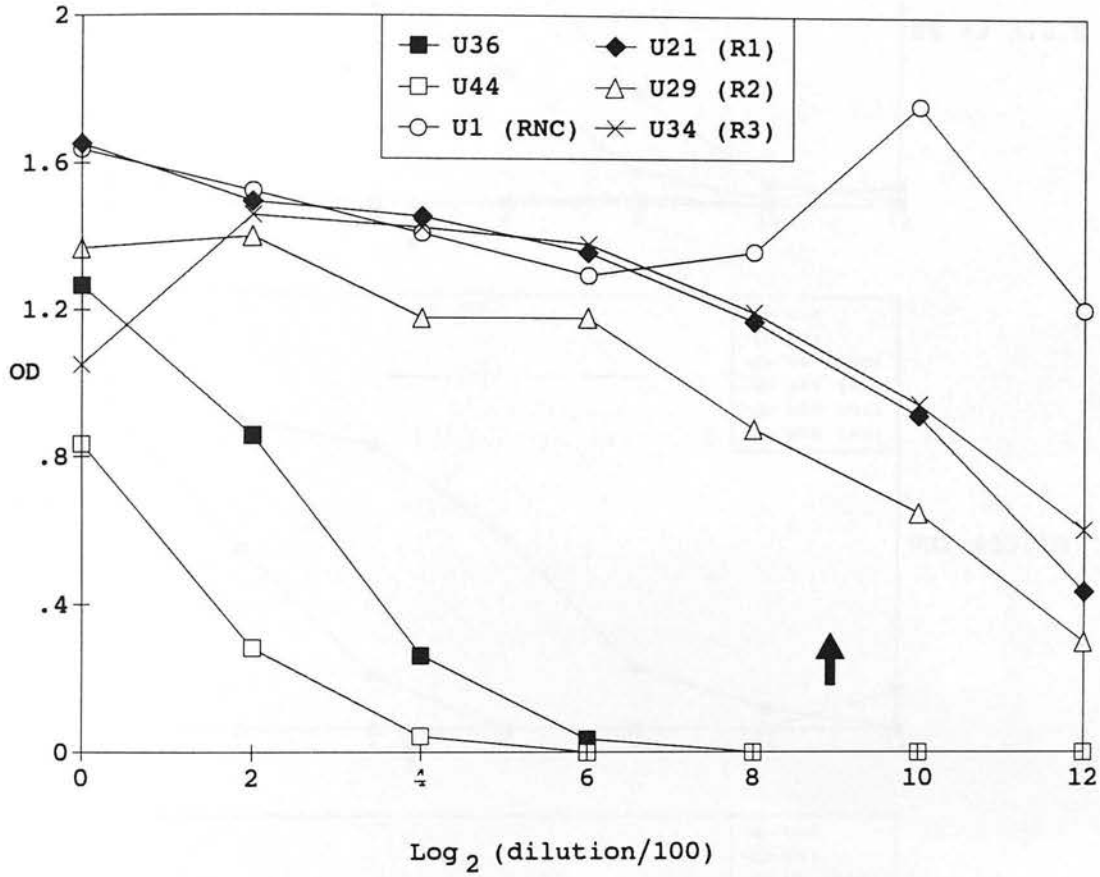


Figure 6.8 Binding (ELISA OD) of a range of dilutions of MAb SZ 27 150.3 (purified preparation) to heat-killed cells of six different *E. coli* isolates from urine. The MAb preparation had a nominal concentration of 2.5 mg/ml. Arrow indicates dilution equivalent to that used in core-typing experiments.

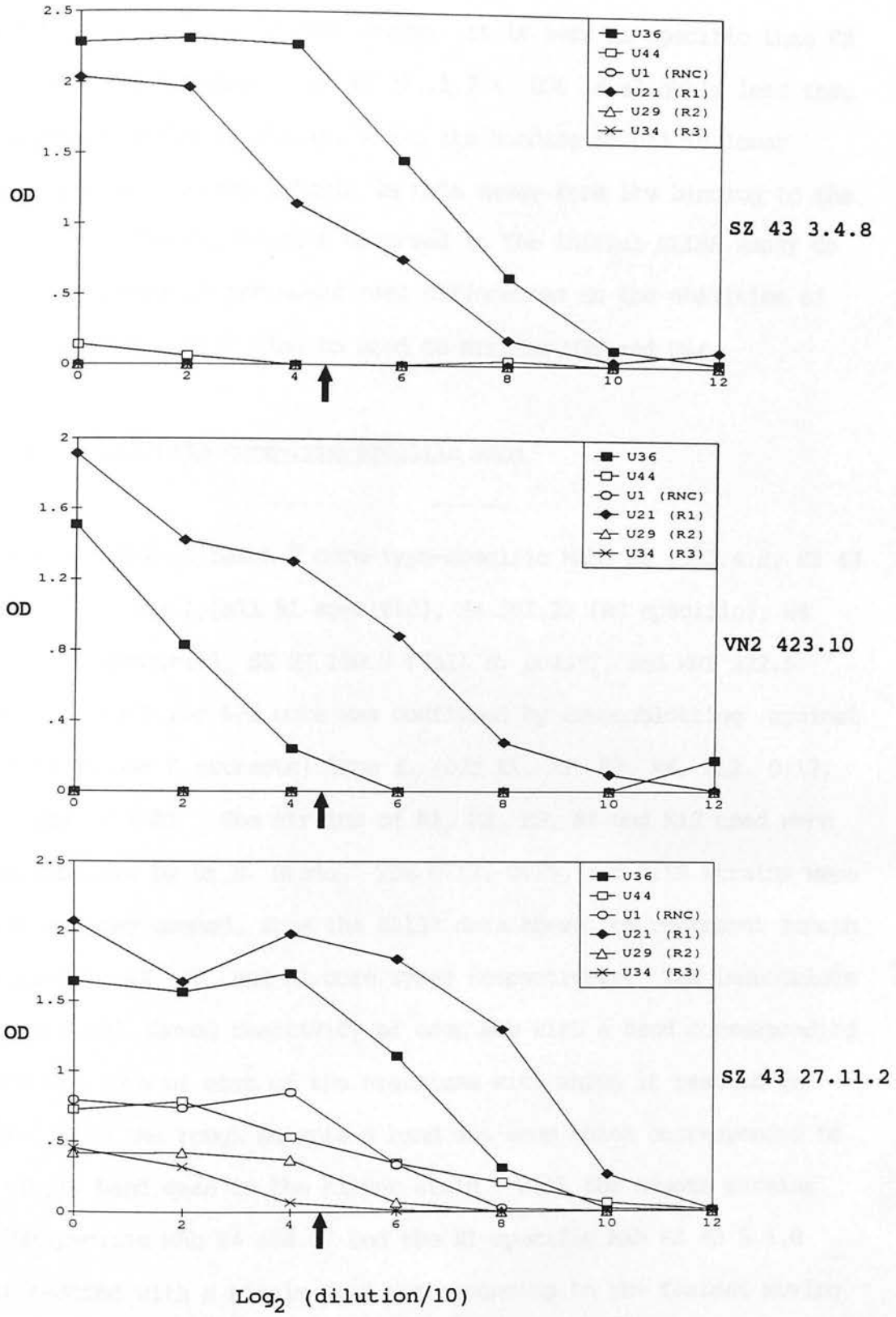


Figure 6.9 Binding (ELISA OD) of a range of dilutions of three R1-specific MAbs (culture supernatants) to heat-killed cells of six different *E. coli* isolates from urine. Arrows indicate dilution equivalent to that used in core-typing experiments.

which has shown that, in some assays, it is less R1-specific than SZ 43 3.4.8. The binding of SZ 43 27.11.2 to U36 is slightly less than its binding to the R1 strain, while its binding to U44 is lower still, and not distinguishable in this assay from its binding to the RNC strain. The differences observed in the initial ELISA assay do therefore appear to represent real differences in the abilities of the three R1-specific MAbs to bind to strains U36 and U44.

Immunoblotting with core-type-specific MAbs

The specificity of batch 2 core-type-specific MAbs SZ 43 3.4.8, SZ 43 27.11.2, VN2 423.1 (all R1 specific), H4 361.23 (R2 specific), W4 434.07 (R3 specific), SZ 27 150.3 ("all *E. coli*"), and WN1 222.5 ("all *E. coli*") for LPS core was confirmed by immunoblotting against LPS (proteinase K extracts) from *E. coli* R1, R2, R3, R4, K12, O:12, O:15, and O:18K1. The strains of R1, R2, R3, R4 and K12 used were those supplied by Dr H. Brade. The O:12, O:15, and O:18 strains were chosen as they seemed, from the ELISA data above, to represent smooth strains with R2, R3, and R1 core types respectively. The immunoblots (figure 6.10) showed reactivity of each MAb with a band corresponding to the LPS core of each of the organisms with which it reacted in ELISA. With the rough mutants a band was seen which corresponded to the single band seen in the silver stain. With the smooth strains the R3-specific MAb W4 434.07 and the R1-specific MAb SZ 43 3.4.8 again reacted with a single band corresponding to the fastest moving band seen in the silver stain, which consists of unsubstituted LPS core. MAb SZ 43 3.4.8 reacted in the same way in blots with the O:1, O:2, O:4, O:6 and O:75 strains, and the other R1-specific MAbs (SZ 43

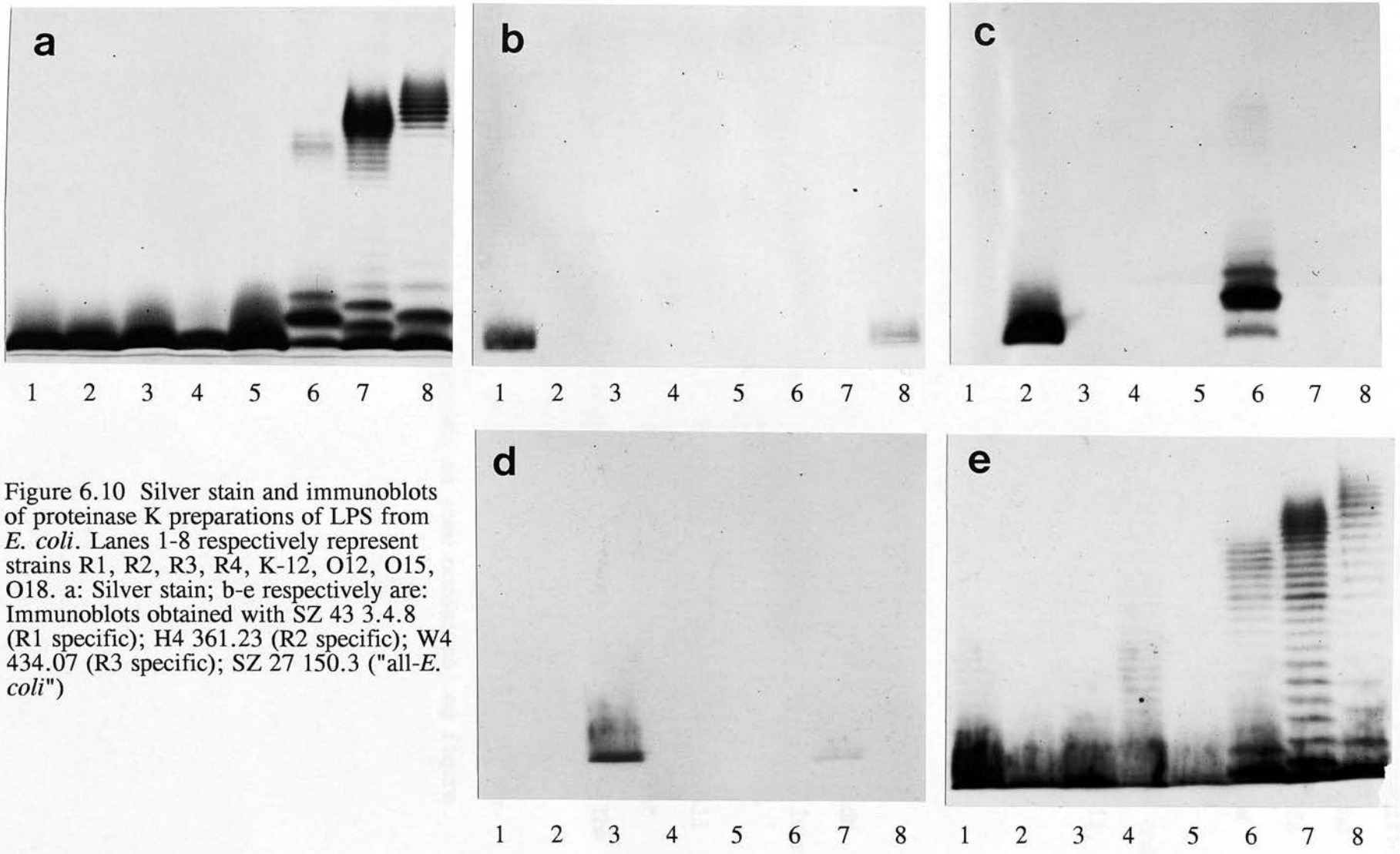


Figure 6.10 Silver stain and immunoblots of proteinase K preparations of LPS from *E. coli*. Lanes 1-8 respectively represent strains R1, R2, R3, R4, K-12, O12, O15, O18. a: Silver stain; b-e respectively are: Immunoblots obtained with SZ 43 3.4.8 (R1 specific); H4 361.23 (R2 specific); W4 434.07 (R3 specific); SZ 27 150.3 ("all-*E. coli*")

27.11.2 and VN2 423.10) produced identical results . The R2-specific MAb H4 361.23 and the all *E. coli* MAb SZ 27 150.3 reacted with the unsubstituted core band and also with the "ladder" pattern of bands corresponding to O-substituted LPS core. A further immunoblot was carried out later with the R2 specific MAb H4 361.23, using additional strains identified (see below) as a) R2 and O:75 b) R1 and O:75 and c) R2 of unknown O type. The result, shown on figure 6.11, confirms that the "ladder" pattern observed with MAb H4 361.23 is restricted to the R2 core type, and is not related to the O:12 antigen or to the O:75 antigen.

MAb SZ 27 150.3 reacted in the blot shown in figure 6.10 with bands in the R1 and R4 LPS preparations which are not visible in the silver stain. These bands may represent either non-covalent polymers of rough LPS [21] or the production, due to a leaky mutation, of small quantities of O-substituted LPS. Alternatively they may represent LPS core substituted with enterobacterial common antigen [121]. The failure to observe these bands in the silver stain may be due to inadequate staining. Material in this region was detected on the silver-stain of similar preparations on some occasions, eg figure 5.14.

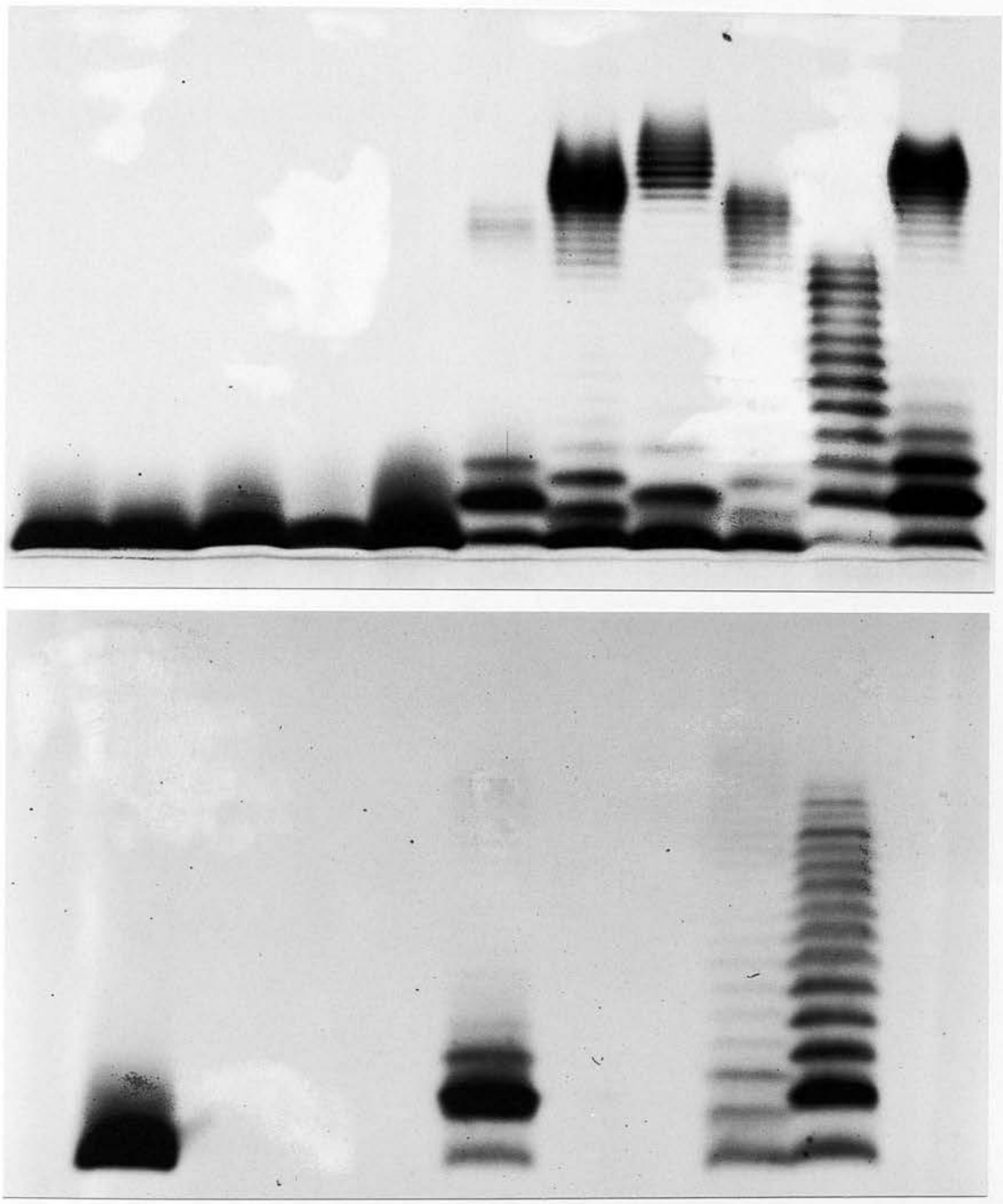


Figure 6.11 Silver stain (top) and immunoblot with R2 specific MAb H4 361.23 (bottom) of polyacrylamide gel of proteinase K preparations of *E. coli*. Lanes 1-8 respectively represent strains R1, R2, R3, R4, K-12, O12, O15, O18. Lanes 9-11 represent blood culture isolates: lane 9: 10b145 (R2, not O typed); lane 10: 10b149 (R2, O:75); lane 11: 9b25 (R1, O:75).

Frequencies of LPS-core types among clinical isolates of *E. coli*

The numbers of wild-type strains falling into the four MAb-defined groups are given in table 6.2. U36 and U44, the two strains which did not bind the "all *E. coli*" MAbs, have been included in the RNC group. R1 strains were the most common overall (123/181, 68%), and were more common among the strains from urine (62/80, 78%) than among those from blood-culture (48/79, 61%, $p = .017$) or faeces (11/21, 52%, $p = .030$).

Relationship between MAb-defined core type and sensitivity to rough-specific phage and K-specific phage.

This is summarized in table 6.3. The collection of wild-type *E. coli* was tested for sensitivity to the rough-specific phages which have been used to distinguish between the different R types of *E. coli* [208], since it was thought that the pattern of reactivity might support the MAb-defined R typing scheme. Fifty of the 180 strains tested were sensitive to one or more of the rough-specific phage. They were found among each of the R-types defined with MAbs. C21 sensitivity, a marker for the R1 rough mutant, was found in 19 strains, 17 of which were R1 and 2 RNC strains. F0 sensitivity is a marker for R2 rough mutants, but no strains were found to be F0 sensitive. There was no other discernible relationship between R type and sensitivity to particular rough-specific phages.

Forty-six of the 180 strains were sensitive to one of the two K-specific phages. The R1 strains accounted for 19 of the 22 K1 strains

Table 6.2 Frequency of core types in wild-type isolates of *E. coli* determined by binding of core-type-specific MAbs in ELISA.

	Number(%) of strains assigned to MAb-defined core types				
Source	R1	R2	R3	RNC[a]	Total
Blood culture	48(61)	6(7)	12(15)	13(16)	79
Urine	64(81)	4(5)	4(5)	8(10)	80
Faeces	11(52)	4(19)	2(10)	4(19)	21
Total	123(68)	14(8)	18(10)	25(14)	180

[a]NC = Not Classified.

Table 6.3. Sensitivity of 180 *E. coli* of different core types to rough-specific, K1-specific, and K5-specific phage.

	Number of strains sensitive to each phage							
Core type (n)	FO	BR10	C21	6SR	T4	Total R+[a]	K1	K5
R1 (123)	0	8	17	5	28	40	19	20
R2 (14)	0	2	0	1	3	3	0	1
R3 (18)	0	0	0	0	2	2	0	1
RNC[b] (25)	0	3	2	2	3	5	3	2
Total (180)	0	13	19	8	36	50	22	24

[a] Total R+ = number of strains sensitive to one or more of the rough-specific phage.

[b] NC = Not Classified.

and 20 out of the 24 K5 strains found. This was not a statistically significant association, however, because of the overall preponderance of R1 strains.

Relationship between MAb-defined core type and O type among the urine and blood-culture isolates.

The urine and blood-culture isolates of *E. coli* were tested for 12 O antigens found commonly among *E. coli* from blood-culture [52,177] or urinary infection [89], as described in the Note at the end of this chapter. The relationship between O types and core types is shown in table 6.4. The proportion of strains which belonged to one of the O-types tested for was higher among those of R1 core type (66/112, 58%) than among the other core types (11/47, 23%, $p = .0005$). Some constant associations between O type and core type are apparent, eg all 26 O:6 strains are R1, and all three O:15 strains were R3. In contrast, O:75 strains were found among both the R1 and the R2 core types.

The proportion of the R1 strains which belonged to one of the common O types was greater among the blood-culture isolates (32 O typed strains among 48 R1 strains, 67%) than among the urine culture isolates (34/64, 53%). This difference is not statistically significant ($p = .177$), but the number of R1 strains not belonging to the common O types could account for the greater proportion of R1 strains among the isolates from urine.

Table 6.4 O type of *E. coli* of different core types from blood and urine cultures.

	Number of each core type associated with the O-type given										
Core type(n)	O1	O2	O4	O5	O6	O7	O8	O15	O18	O75	NT[a]
R1 (112)	11	0	6	1	26	3	3	0	10	6	46
R2 (10)	0	0	0	0	0	0	0	0	0	3	7
R3 (16)	0	0	0	0	0	0	0	3	0	0	13
RNC[b] (21)	1	2	0	0	0	2	0	0	0	0	16
Total (159)	12	2	6	1	26	5	3	3	10	9	82

[a] NT = Not Typed by the available O-specific sera.

[b] NC = Not Classified

Relationship between R type, serum resistance, and LPS chemotype among blood-culture strains.

Serum resistance, which is a property of most invasive strains of *E. coli* [52] was determined for all the blood-culture isolates. Of the 79 strains, 21 (26%) were serum sensitive. Serum sensitive strains were present in all the core types but were slightly less common among the R1 strains (9/48, 19%) than among the other R types (10/31, 32%). This difference may have been due to the association of R1 core with K1 and K5 capsule, since all of the 11 K1 strains and all but one of the 10 K5 strains from blood-cultures were serum resistant.

Seventy of the 79 blood-culture strains produced a typical "ladder" pattern LPS in silver stains of polyacrylamide gels of proteinase K digests. Typical "ladder" patterns were produced by some of the strains which were sensitive to one or more rough-specific phage, as was found by Cross et al [52]. The other nine strains showed only one or a few fast-moving bands, one corresponding to the unsubstituted core. Seven of these strains, together with some strains producing typical "ladder" patterns are shown in figure 6.12. All these 9 strains were sensitive to one or more of the rough-specific phage. Three of the nine were R1, three were R2, two were R3 and one was RNC. One was O typeable, an O:1 K1 strain. Two were serum resistant, both K1 positive strains.

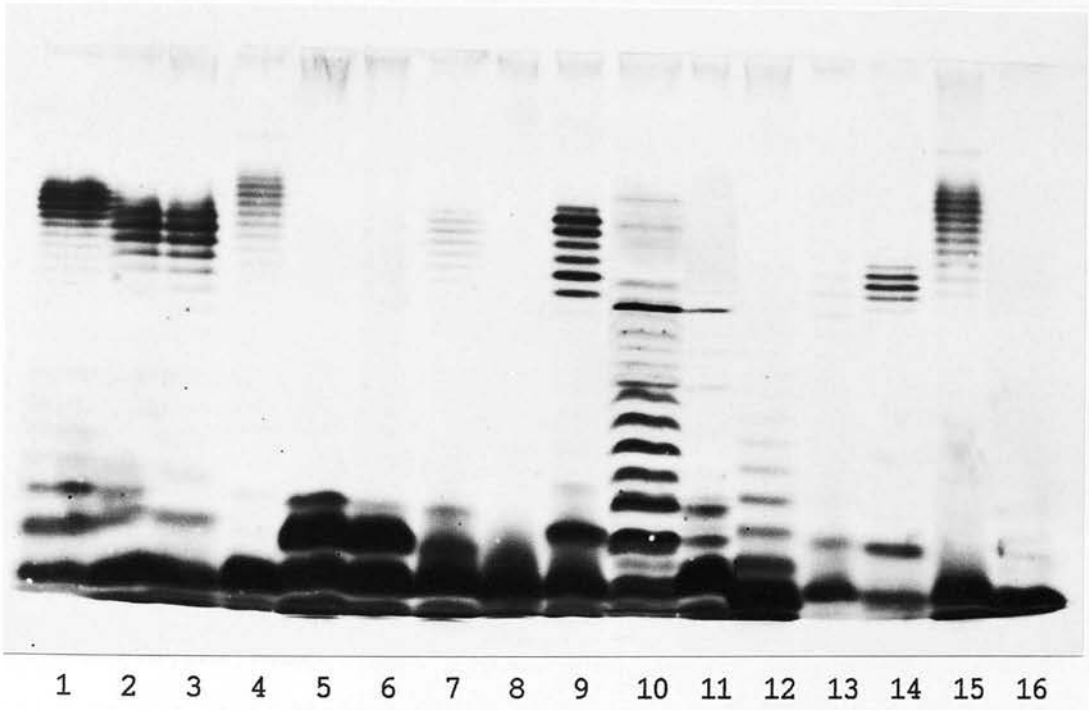


Figure 6.12 Silver stains of polyacrylamide gel of LPS (proteinase K digests) from some blood-culture isolates of *E. coli*. The strains in lanes 5, 6, 8, 11, 12, 13, and 16 were considered to show an atypical pattern lacking the usual "ladder" of high molecular weight bands, and were all sensitive to one or more rough-specific phage. The strains represented by lanes 1, 3, 4, 9, and 15 were also sensitive to rough-specific phage. The strain in lane 10 is 10b149 which shows a unique ladder pattern dominated by low molecular weight bands. This strain is also seen in figure 6.11.

Binding of anti-LPS-core MAbs to Enteropathogenic *E. coli* and *Shigella* spp.

The "all *E. coli*" MAb SZ 27 150.3, and individual core-specific MAbs from batch 2 (R1: VN 2 423.10, R2: H4 361.23, and R3: W4 434.07) were used to test *Shigella* and enteropathogenic *E. coli* strains. These experiments were carried out in two separate batches, each including *E. coli* rough mutants R1, R2, R3 and R4 as controls. Table 6.5 summarises the results. The OD results have been classified as positive or negative, with a positive taken as more than 60% of the result obtained with the appropriate rough control organism. In most cases the wild-type strains gave OD results close to or higher than those obtained with the rough strain.

Both of the *Shigella sonnei* strains reacted with the R1-specific MAb, while the two *Shigella flexneri* strains reacted with the R3-specific MAb. These strains also reacted with the "all *E. coli*" MAb. These results are consistent with previous studies of LPS core in these species [78,101,145]. The two *Shigella dysenteriae* type 2 strains also reacted with the R3-specific MAb, but they failed to react with the all *E. coli* MAb SZ 27 150.3, implying that they probably have a different core structure. Enteropathogenic *E. coli* strains classified as R1, R2, R3 and RNC were found, though there was a predominance of R3 strains among the group of strains tested. The finding of two different core types among the two available strains of *E. coli* O:127 was surprising in view of the suggested "clonal" nature of enteropathogenic *E. coli* [178], but may reflect the suggestion that many sporadic isolates of enteropathogenic serotypes of *E. coli* are not true enteropathogenic strains [117].

Table 6.5 Core-typing of *Shigella* spp. and enteropathogenic *E. coli* strains with MAbs in ELISA.

Strain	Reaction[a] with MAbs[b] specific for *E. coli* core types				Core type as defined by MAbs
	All	R1	R2	R3	
Shigella sonnei 4/2	+	+	-	-	R1
S. sonnei 2/1	+	+	-	-	R1
S. flexneri 3/1	+	-	-	+	R3
S. flexneri 1A	+	-	-	+	R3
S. dysenteriae type 2[c]	-	-	-	+	RNC
E. coli 0125[d]	+	+	-	-	R1
E. coli 0126[d]	+	-	-	+	R3
E. coli 0127[d]	+	-	+	-	R2
E. coli 0127[e]	+	-	-	-	RNC
E. coli 0128[f]	+	-	-	+	R3
E. coli 0157[g]	+	-	-	+	R3

Notes. [a] "+" indictates an OD > 60% of that obtained with the appropriate R type control organism; "-" indicates an OD of < 10 % of the appropriate control. [b] The MAbs used were SZ 27 150.3 (reactive with all *E. coli* core types) VN2 423.10 (R1 specific) H4 361.23 (R2 specific) and W4 434.07 (R3 specific). [c] Two stock strains. [d] Stock culture. [e] Recent sporadic clinical isolate. [f] One stock strain and three clinical isolates. [g] Six clinical isolates.

Binding of MAbs to Gram-negative bacilli other than *E. coli* or *Shigella* spp.

Blood-culture isolates of other Gram-negative genera (*Acinetobacter*, 7; *Citrobacter*, 5; *Enterobacter*, 18; *Klebsiella*, 14; *Proteus*, 9; *Providencia*, 1; *Pseudomonas*, 8; and *Salmonella*, 2; *Serratia*, 5) were also tested in ELISA with the full panel of batch 2 MAbs. The only positive reaction observed with the "all *E. coli*" MAbs or core-type-specific MAbs was between the two *Salmonella* strains and the "all *E. coli*" MAbs. As indicated above, some other MAbs were identified which reacted with *E. coli* R2 and *Salmonella* or with *E. coli* K12 and *Salmonella*. Three MAbs were identified which did apparently cross-react with a wider range of bacterial species, and these are discussed further in chapter 7.

DISCUSSION

I have described a group of MAbs that bind to the LPS core of *E. coli* in polymyxin-LPS ELISA (data not shown) and in immunoblots, and studied their binding to a wide range of heat-killed Gram-negative bacteria in ELISA. Some of the MAbs bind to *Salmonella* and *Shigella* strains which share *E. coli* core structures. The MAbs do not bind to a range of other Gram-negative bacilli, confirming that the binding observed is specific.

MAbs which bind to rough *E. coli* of one core type and which also bind to some smooth *E. coli* have been used to identify three mutually exclusive groups among *E. coli* isolates. These groups have been named R1, R2, and R3 after the corresponding rough mutants. A fourth group, which could not be assigned to a particular core type, has been referred to as RNC, and is probably of heterogeneous core type. I have included in the RNC group the strains which did react with one or more of the R1-specific antibody but which did not react with the "all *E. coli*" MAb. If these strains had been of R1 core type then a reaction with the "all *E. coli*" MAb would have been expected.

E. coli strains assigned to the groups R1, R2 and R3 defined with MAbs may have the core structure of the named rough strain, though this has not been confirmed by chemical analysis.

Results reported in chapter 5 suggested that there may be some heterogeneity within the R1 group. All five *E. coli* strains used in chapter 5 were subsequently assigned to the R1 group on the basis of

experiments with the MAbs described in the current chapter (data not shown). MAbs H4 351.18 and H7 41.76 (table 5.3) failed to bind (or bound very weakly) to *E. coli* strain 8b317 in immunoblots, but did bind to the other four *E. coli* strains tested. It was this observation which led to the idea of looking for core-type-specific MAbs, but which now suggests some heterogeneity in the R1 group. There are other possible explanations however, including differences in the amount of LPS present in the preparations used in chapter 5, and problems with transfer of LPS to nitrocellulose at one end of the blotting tank. The whole-cell ELISA experiments in chapter 5 (figures 5.2 and 5.3) did not clearly demonstrate a difference between *E. coli* strain 8b317 and the other *E. coli* strains with respect their binding of MAbs H4 351.18 and H7 41.76.

The presence of C21 sensitivity (a marker for R1 rough mutants) among the wild-type strains of MAb-defined R1 rather than R2 or R3 core type supports the view that the MAb-defined core types are the same as the reference rough strains. Two RNC strains were also C21 sensitive, but the specificity of these phages for particular core types is not based on an independent knowledge of their receptor sites, and it is not known how they might react with as yet unidentified core types of *E. coli*. Phage typing is of little value in determining the core type of wild-type strains since most wild-type strains are resistant to all of the phage used.

No strains of R4 or K12 core type have been positively identified. This may be explained by the lack of suitable specific MAbs. We did have MAbs reactive with K12, but these also reacted with R2, and did

not bind to a clearly defined group of wild type strains other than those which reacted with the R2-specific MAbs. The RNC group may include strains with R4 or K12 core, but probably also contains other as yet unidentified core types. The RNC group is heterogeneous, as seen from the variable reactivity with those MAbs that react with K12, and the lack of reactivity with the "all-*E. coli*" MAb observed with urine strains U36 and U44.

MAbs reactive with complete core structures have been reported by others. Tsang and colleagues reported MAb T6 [235] which reacts with the LPS core of *Salmonella* and the *E. coli* R2 rough mutant, and MAb 105 [236] which reacts with the core of *Salmonella* only. Luk et al [133] described two other MAbs (Ty1 and Ty2) which recognise the complete core of *Salmonella*. Ty1 reacted only with unsubstituted core, and did not react with any of the *E. coli* core types. Ty2 reacted with O-substituted core of *Salmonella* and also reacted with the *E. coli* R2 rough mutant. It was not tested against smooth *E. coli* other than O111, which is of R3 core type [207]. Nnalue et al [170] described MAb MM3 which also reacted with *Salmonella* and with *E. coli* R2 core but not with other *E. coli* core types. MM3 also reacted with 10 of 68 clinical isolates of *E. coli*, and reacted with O-substituted *Salmonella* core in immunoblots.

Viret et al [245] described MAb Sh9R which binds *E. coli* R1 core but not other *E. coli* cores. It does not bind to *Salmonella*, but does bind to *Shigella sonnei* and to other *Shigella* species which are reported to have R1 core. It does not bind to O-substituted core in immunoblots. Attempts to define its epitope by competition studies

were unsuccessful.

Comparison of the structural formulae (figure 1.4) with the specificities of the core-type-specific and "all *E. coli*" MAbs could suggest binding of the MAbs to particular sugar residues. R1 and R3 cores each have a unique terminal branching hexosyl unit (β1-3 linked glucose and α1-3 linked N-acetyl glucosamine respectively) which may be the epitope for the R1 and R3 MAbs. Viret et al [245] also concluded that MAb Sh9R recognised the terminal glucose residue. *E. coli* R2 and *Salmonella* core share a terminal α1-2 linked N-acetyl glucosamine, while R2 and K12 have in common the next three sugar residues. These may therefore be the epitopes for the MAbs which bound to these combinations of strains. Competition studies have shown however that the epitope of the R2/*Salmonella* MAb Ty2 [133] was in the Glucose-Galactose-Glucose backbone of the core, and that the epitope of the apparently similar MAb MM3 [170] was in the heptose region, where there are no differences in the accepted structural formulae.

A unique binding site for the R2-specific antibody is not obvious from the structural formulae but, as is apparent from the results of competition studies discussed above the structural formulae probably give an incomplete picture of the structure of LPS-core. From the calculated three-dimensional structure of the different core types [103] however it appears that the branch-terminating α1-6 galactose present in R2, *Salmonella* and K12 may be in a different orientation in R2, and may therefore be the R2-specific epitope. It is of note that, while most of the core-specific MAbs reacted in immunoblot only

with unsubstituted core, the R2-specific MAb also reacted with O-substituted core, giving a ladder pattern in immunoblots. This implies that the R2-specific epitope is the only one not altered or masked by the attachment of the O polysaccharide. Further study of the binding site of these antibodies may therefore be useful in elucidating the three-dimensional structure of LPS core, and in attempts to define the attachment site of the O polysaccharide.

The observation that the urine strains U36 and U44 showed differential binding of the three "R1-specific" MAbs implies that these three R1-specific MAbs have different specificities, indicating that the R1-specific region of *E. coli* R1-type LPS-core contains three distinct, though perhaps overlapping, epitopes. All three epitopes are apparently present on the R1 group of organisms, and all are lost in O-substituted LPS. U36 strongly expresses the epitopes recognised by SZ 43 3.4.8 and SZ 43 27.11.2. U44 and some other *E. coli* strains bind SZ 43 27.11.2 to a lesser extent, suggesting the presence of an epitope which is similar to but not identical with the epitope recognised by this MAb on *E. coli* R1. U36 binds VN2 423.10 only at high MAb concentration, again suggesting the presence of an epitope similar to but not identical with the epitope recognised on *E. coli* R1.

U36 and 44 would be an interesting starting point for further study of the detailed structure of *E. coli* R1 core, and it is unfortunate that U44 is no longer viable. Some doubt must remain over whether U44 is really an *E. coli*, since it was not possible to recheck its identification. As far as the interpretation above of the

differential binding of the three R1-specific MAbs is concerned, it does not matter if the organism is not an *E. coli*.

It is likely that the R2- and R3-specific regions are of similar complexity to the R1-specific region, and possibly as complex as the region or regions common to *E. coli* R2 and *Salmonella* [133,170]. Demonstrating the presence of multiple epitopes requires a suitable collection of MAbs with subtly different specificities. We did not appear to have such a collection of MAbs, but MAbs of interest in this regard may have been overlooked because of problems in production and labelling of some of the R2- and R3-specific MAbs. In order to demonstrate multiple epitopes by the methods employed here one would also require a collection of organisms which express suitable variants of the core region. Such strains may be very rare or non-existent. Alternative and more systematic approaches would be to look at binding to synthetic analogues of core structures and to test for competitive inhibition between the different MAbs.

The binding of the "all *E. coli*" MAb SZ 27 150.3 to almost all of the *E. coli* strains and to the *Salmonella* and *Shigella* strains, together with the pattern of binding of the core-specific MAbs, supports the view that these organisms share closely related LPS cores. The lack of binding of any of the antibodies to other genera suggests that these core structures are not distributed more widely. The structure of LPS core has not been determined for all the other genera, but it has been shown that the LPS cores of *Citrobacter* [197,198] *Proteus* [192] and *Pseudomonas* [200] are different from that of *E. coli*.

R2 core type accounted for 8% of the collection of *E.coli* strains reported here. A similar overall frequency of R2 core type is suggested by Nnalue et al [170] who reported that MAb MM3 (which reacted with *Salmonella* and *E. coli* R2 core) bound to 10 (14.7%) of 68 clinical isolates of *E. coli*. The overall figure reported by Nnalue et al [170] masks a major difference between faecal and blood-culture isolates of *E. coli* however, since 10 of 36 faecal isolates reacted with MM3, while none of 32 blood culture isolates reacted. It is not clear from this report whether the faecal isolates were enteropathogenic serotypes of *E. coli* or simply commensal strains. MAb T6, which also recognises the R2 core of *E. coli* and the core of *Salmonella* spp, did not react with 25 clinical isolates of *E. coli* in Hong Kong [235]. These differences in the apparent frequency of R2 core may represent differences in the specificity of the MAbs as well as geographical and temporal differences in the true frequency of R2 core. A capture ELISA using MAb T6 which is intended to detect *Salmonella* in clinical samples has been described [41]. This assay may yield a significant number of false positives if *E. coli* of R2 core type is as common as is suggested by the data reported here.

The tendency for certain apparently distinct properties of *E. coli* to occur together [178] seems to apply to the relationship between core types and O and K types. The association between core type and O type reported here is largely in agreement with the results of Viret et al [245] who found that the R1-specific MAb Sh9R reacted with *E. coli* serotypes O:1, O:6, O:7, O:18 and O:75 but not with O:12 or O:15. It may be that there are chemical or structural factors limiting the ability of *E. coli* to express certain combinations of O

types and core type (eg 0:15 and R1), or it may be that some such combinations have a selective advantage in the host. The finding that there was greater proportion of R1 core type among the urine strains, and in particular that there was a greater proportion of R1 strains not belonging to one of the common O types, may indicate some relationship between R1 core type and unusual O types and pathogenicity in the urinary tract. Further studies are needed before firm conclusions can be drawn on this point.

Core typing with MAbs may be a useful addition to current typing methods for *E. coli*. The number of possible types is small, and the core type does not usually discriminate among strains of a given O and K type, but core typing may be useful in recognising relatedness between groups of *E. coli*. It would be of interest to use the MAbs described here to investigate the LPS-core type of a wider range *Shigella* and *E. coli* from a variety of sources.

NOTE ON THE O-TYPING METHOD USED.

Small amounts of Difco absorbed antisera to *E. coli* O antigens O:1, O:2, O:4, O:6, O:7, O:12, O:15, O:18, O:22, O:75 and O:86 were a gift from Dr CC Blackwell, of the Department of Medical Microbiology, Edinburgh University. Using these antisera the standard method of O typing *E. coli* strains was adapted, by including an ELISA based initial screening stage, to reduce the workload and amount of serum required.

An initial experiment was devised to determine the feasibility of this approach, and to determine the appropriate dilution of serum to use. NUNC polysorb strips were coated with heat-killed *E. coli* strains O:4, O:12 and O:15 and post coated as described in materials and methods, and an ELISA assay was performed using varying dilutions of absorbed rabbit antisera(Difco) to *E. coli* O antigens 4, 12 and 15. On the basis of these results (figure 6.13) a serum dilution of 1 in 1000 was chosen for the assay of the full panel of organisms, since this seemed to give a minimal OD with the heterologous strains tested while maintaining a reasonably high homologous OD. If necessary the incubation of the substrate could be continued for a longer period to achieve a higher specific OD.

The full panel of 79 blood culture *E. coli* and standard strains was tested in ELISA with a 1 in 1000 dilution of the available Difco absorbed O typing sera. ODs were recorded at 60 min and 90 min incubation. The 60 min readings were used for all antisera except O:18. In the case of the O:18 antiserum a printer fault caused the

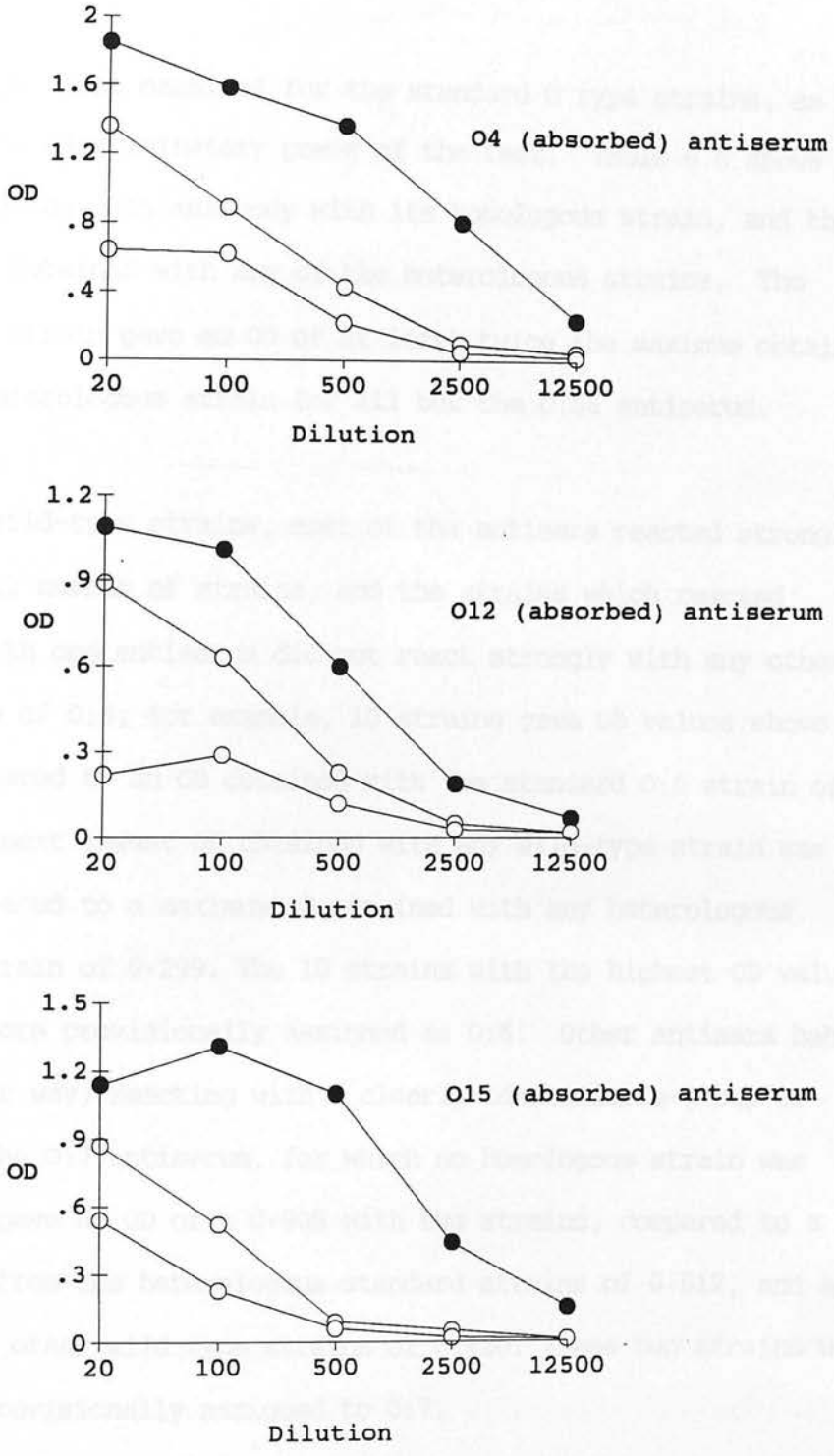


Figure 6.13 Binding (ELISA OD) of absorbed rabbit anti-O antisera to *E. coli* O:4, O:12, and O:15. ● = homologous strain, ○ = heterologous strains.

loss of most of the 60 min data for the blood-culture isolates, and the 90 min data were therefore used.

Results were first examined for the standard O type strains, as an index of the discriminatory power of the test. Table 6.6 shows the OD obtained for each antibody with its homologous strain, and the highest OD obtained with any of the heterologous strains. The homologous strain gave an OD of at least twice the maximum obtained with any heterologous strain for all but the O:86 antiserum.

Among the wild-type strains, most of the antisera reacted strongly with a small number of strains, and the strains which reacted strongly with one antiserum did not react strongly with any other. In the case of O:6, for example, 10 strains gave OD values above 0·978, compared to an OD obtained with the standard O:6 strain of 0·937. The next lowest OD obtained with any wild-type strain was 0·360, compared to a maximum OD obtained with any heterologous standard strain of 0·299. The 10 strains with the highest OD values were therefore provisionally assigned as O:6. Other antisera behaved in a similar way, reacting with a clearly identifiable group of strains. The O:7 antiserum, for which no homologous strain was available, gave an OD of ≥ 0·908 with two strains, compared to a maximum OD from the heterologous standard strains of 0·512, and a maximum for other wild-type strains of 0·420. These two strains were therefore provisionally assigned to O:7.

Conventional tube agglutination was carried out to confirm the identification of all the provisionally identified O:18 and O:7

Table 6.6 ELISA results obtained with Difco absorbed anti-O sera tested at a dilution of 1 in 1000 against a panel of standard smooth strains of *E. coli* of known O type[a].

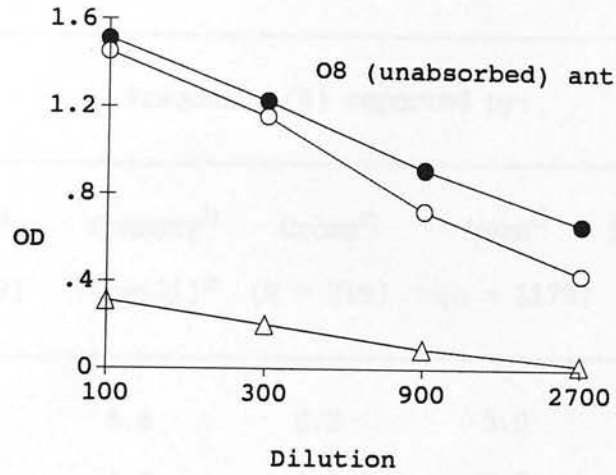
	OD obtained with			
	Homologous strain	Heterologous strains		
Antiserum		Maximum	Minimum	Discriminatory ratio[b]
0:1	0.803	0.269	0.137	2.99
0:2	0.978	0.222	0.094	4.41
0:4	0.826	0.152	0.036	5.43
0:6	0.937	0.299	0.122	3.25
0:7	N/A[c]	0.512	0.210	N/A
0:12	0.396	0.161	0.056	2.46
0:15	0.837	0.365	0.047	2.29
0:18	0.837[d]	0.416	0.110	2.01
0:22	N/A	0.122	0.037	N/A
0:75	0.893	0.331	0.126	7.09
0:86	0.258	0.273	0.090	0.95

Notes [a] The standard strains used were O:1, O:2, O:4, O:6, O:8, O:12, O:15, O:18, O:75, and O:86. [b] The discriminatory ratio was defined as (OD obtained with homologous strain)/(Maximum OD obtained with any heterologous strain). [c] N/A = not available. [d] The lowest result obtained with any of the O:18 strains is given.

strains, and on the strain with the lowest OD from each of the other provisionally identified O types.

Absorbed O:8 and O:9 antisera were not available, and no commercial source of antisera to the *E. coli* O antigens found commonly in blood and urine cultures could be found. Unabsorbed antisera to O:8 and O:9 was subsequently obtained from the Statens Seruminstitut, Copenhagen. However, these antisera were not able to distinguish between homologous and heterologous O-types of *E. coli* in ELISA as the absorbed sera had done. Figure 6.14 shows an example of the results obtained. Attempts were made to absorb out cross-reactivity, but without success. It was therefore decided to screen the organisms with these sera by conventional tube agglutination using a final serum dilution of 1 in 80, and confirm by determining the highest titre of antiserum which would agglutinate the organism suspension. Four of the blood-culture isolates of *E. coli* were agglutinated by the O:8 antiserum at 1 in 80, of which 3 were agglutinated at 1 in 1280, the same titre as the control organism. None of the blood-culture isolates were agglutinated by the O:9 antiserum.

E. coli strains from urine cultures were O-typed in the same way as the blood-culture isolates had been. The results of O typing are shown in tables 6.4, 6.7, and 6.8. Grüneberg et al [89] noted that there were statistically significant differences between the numbers of strains of particular O types reported in different series of urinary infections, even from different centres within a few miles of one another in London. The differences were minor however, and a



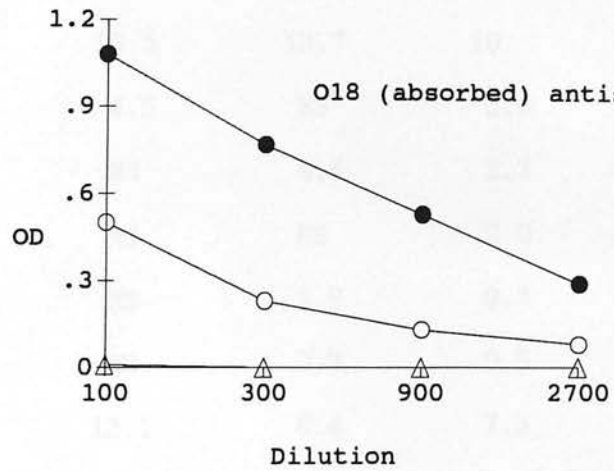


Figure 6.14 Binding (ELISA OD) of absorbed and unabsorbed anti-O antisera to E. coli O:8 and O:18. ● = homologous strain; ○ = heterologous strain; △ = binding to ELISA wells which had not been coated with *E. coli* but which had been post-coated.

Table 6.7 Frequency of common O types among isolates of *E. coli* from blood cultures.

O type	Frequency (%) reported by:				
	Ørskov[a] (n =539)	Cheasty[b] (n=421)[e]	Cross[c] (N = 219)	Mean[d] (n = 1179)	This thesis (n = 79)
0:1	2.8	6.4	8.2	5.0	8.9
0:2	10.8	4.9	3.2	7.2	1.3
0:4	10.6	5.2	8.2	8.2	3.8
0:6	8.2	10.5	13.7	10	12.7
0:7	3.2	4.5	NS[f]	3.0	2.5
0:8	4.0	NS	4.6	2.7	3.8
0:9	4.2	NS	NS	2.0	0
0:12	NS	NS	1.8	0.3	0
0:15	NS	NS	2.7	0.5	1.3
0:18	3.3	13.1	6.4	7.5	6.3
0:22	3.0	1.2	NS	0.4	0
0:75	7.2	4.7	7.2	5.9	7.6

Notes [a] Ørskov and Ørskov [177]. [b] Cheasty et al [39]. [c] Cross et al [52]. [d] Mean = mean of results from the three published series shown. For the purposes of this calculation it has been assumed that no strains of a given O type were identified in a series where the number is not stated (NS) in the publication. [e] The total given in the paper is 411, but the numbers given add up to 421. [f] NS = not stated in the publication. In the series of Cheasty et al [39] and Ørskov and Ørskov [177] NS means less than five strains of a particular serotype.

Table 6.8 Frequency of common O types among isolates of *E. coli* from urine cultures

O type	Frequency (%) reported by:	
	Grüneberg et al [89] (n = 1771)	This thesis (n = 80)
O:1	7.7	6.3
O:2	4.5	1.3
O:4	11.8	3.8
O:6	11.8	20.0
O:7	5.0	3.8
O:9	1.0	0
O:12	1.1	0
O:15	NS[a]	2.5
O:18	2.9	6.3
O:75	7.6	3.8

[a] NS = Not Stated.

limited range of types (notably 0:1, 0:2, 0:4, 0:6, 0:18, and 0:75) were frequent in all series of urinary infection, as they are in series of blood culture *E. coli*. Within these broad limits, the relative numbers of strains in each of the common O groups reported here is in agreement with that reported by others.

The use of ELISA screening to determine the O types of *E. coli* appeared to work very well, and could probably be used without the need to confirm any O types by tube agglutination. It did however require appropriate absorbed sera. Since these sera are no longer commercially available however it is unlikely that this method will be widely applied.

CHAPTER 7

CROSS-REACTIVE MONOCLONAL ANTIBODIES WHICH DO NOT REACT WITH LPS-CORE.

INTRODUCTION

During the Sandoz project some MAbs were produced which were widely cross reactive in ELISA with Gram-negative bacteria, but which did not react with LPS-core in ELISA. These were considered therefore to be reactive with some bacterial antigen other than LPS-core. Three of these MAbs (WN1 16.1, H7 2.1.5, and H4 250.7) were selected for further study. The MAbs preparations used were all from batch 2.

Experiments with HA-1A (Centoxin) are also considered in this chapter.

ELISA

ELISA with heat-killed bacteria was carried out as described in chapter 6. ELISA with whole bacteria was carried out in the same way, except that the heating of bacteria was omitted.

Outer membrane protein (OMP) preparations

Bacteria were grown up overnight in 100 ml nutrient broth in a shaking incubator. Broth was then centrifuged at 10,000 g for 10 min. The pellet was suspended in distilled water, re-centrifuged resuspended in 5 ml of distilled water, and 0.55 ml of 7% w/v Sarkosyl (Sigma) added. The suspension was then sonicated (Microson ultrasonic cell disrupter, Heat Systems-Ultrasonics Inc., NY, USA) for six 1 min bursts at an amplitude of 10 µm on iced water with intervals of 30 s. The suspension was then centrifuged at 10,000 g for 10 min to remove unbroken cells. The remaining supernatant was centrifuged at 80,000 g for 1 h at 4°C to harvest insoluble outer membranes. The pellet was resuspended (by aspiration into a syringe through a 26-gauge needle) in 4 ml of distilled water, centrifuged again at 80,000 g for 1 h at 4°C. The pellet was then resuspended in 2 ml of distilled water. The protein concentration was measured by the method of Lowry [132]. The preparation was stored at -20°C. Samples containing 25 µg of protein were mixed with an equal volume of double-strength sample buffer and heated to 100°C for 2 min before electrophoresis.

Freeze-dried bacteria (100 mg) were suspended in 2.2 ml distilled water. The following were successively added and mixed: 0.4 ml 100mM Tris HCl pH 8.0, 0.4 ml 0.5 M Mg Cl_2, and 1.0 ml 8% Triton X-100. Finally 1ml ethanol was added, the mixture tightly capped and placed at 100°C for 10 min. After cooling the mixture was centrifuged at 100,000 g for 90 min at room temperature and the precipitate washed once in 4 ml 10 mM Tris HCl pH 8.0 containing 10 mM $MgCl_2$. The precipitate was suspended in 1 ml distilled water, 1ml 0.2 M EDTA pH 8.0, 1 ml 2M NaCl, and 1 ml 8% Triton X-100. The suspension was mixed and incubated with shaking at 37°C for 60 min and then centrifuged at 15,000 g for 15 min at room temperature. The supernatant from this step (3.6 - 4.0 ml) was mixed with 0.4 ml 1M $MgCl_2$, and incubated at 37°C for 60 min. The resultant opaque solution was then centrifuged at 100,000 g for 90 min at 15°C, and the resulting precipitate washed once in 4.0 ml 10 mM Tris HCl pH 8.0 containing 10 mM $MgCl_2$.

Sandoz MAbs

Figures 7.1 - 7.3 show the results of the ELISA screen of the collection of 69 non-*E. coli* strains from blood cultures with the three selected MAbs. H4 250.7 and H7 2.15 produced very similar patterns of reactivity, and were placed together by the correlation-sorting analysis of the results. WN1 16.1 produced a different pattern. Neither MAb H4 250.7 nor H7 2.15 bound to the *Proteus* strains, for example, whereas WN1 16.1 did bind to most of these strains. There were some similarities in the patterns of reactivity of the three MAbs however. For example, all bound to all of the *E. coli* strains, and none bound to any of the *Acinetobacter* strains.

From the strains in the original screen, a panel of bacteria was selected to include organisms from a variety of genera which bound strongly to each MAb. I decided to compare the binding of these MAbs to heat-killed and "whole" (not heat-killed) bacteria in ELISA. The results of the initial experiment are shown in figure 7.4. The reactivity with heat-killed bacteria confirmed the pattern observed previously. MAbs H4 250.7 and H7 2.15 gave almost identical patterns of reactivity. As before, neither reacted with the *Proteus* strains. MAb WN1 16.1 reacted with all of the selected strains. MAb SZ 27 150.3 reacted only with the *E. coli* strain. None of the MAbs reacted with either of two *Acinetobacter* sp. (10b335 and 10b348) or with two *Pseudomonas* spp. (10b411 and 10b224) (not shown). The reactivity with whole bacteria showed major reductions in the binding of MAbs H4

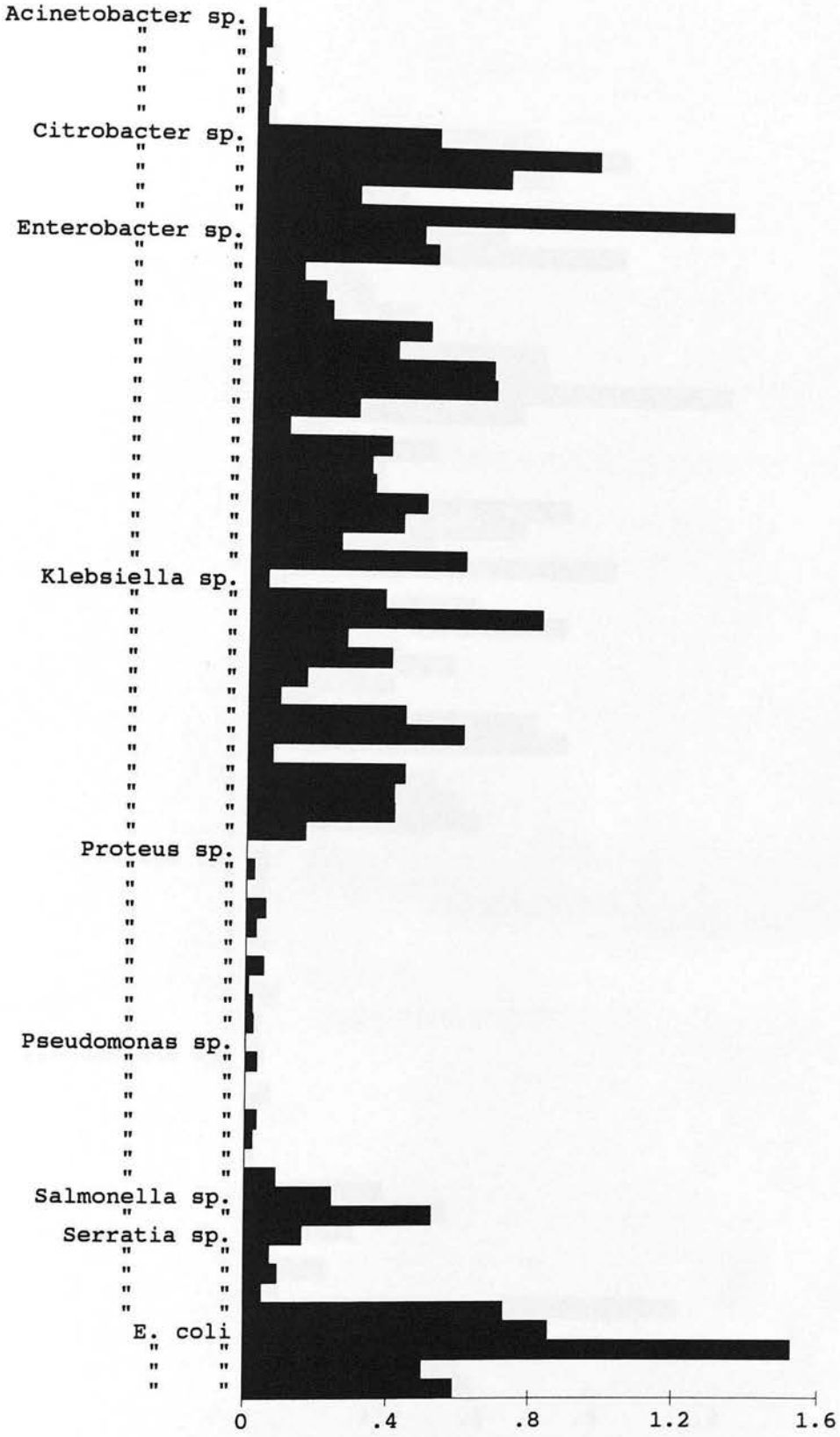


Figure 7.1 Binding (ELISA OD) of MAb H4 250.7 (culture supernatant diluted 1 in 250) to heat-killed cells of 68 blood culture isolates of bacteria other than *E. coli* and four faecal isolates of *E. coli*.

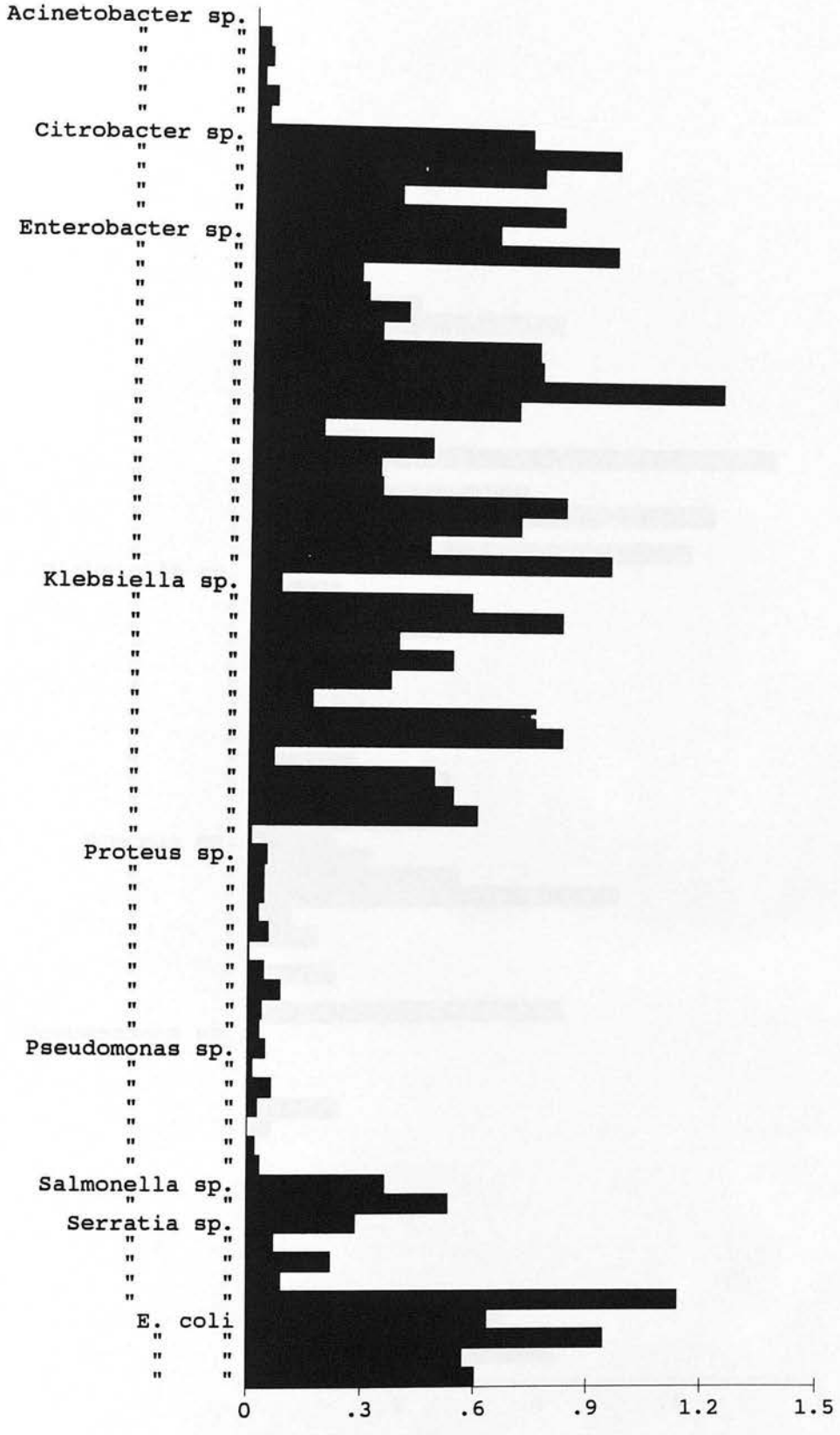


Figure 7.2 Binding (ELISA OD) of MAb H7 2.15 (culture supernatant diluted 1 in 250) to heat-killed cells of 68 blood culture isolates of bacteria other than *E. coli* and four faecal isolates of *E. coli*.

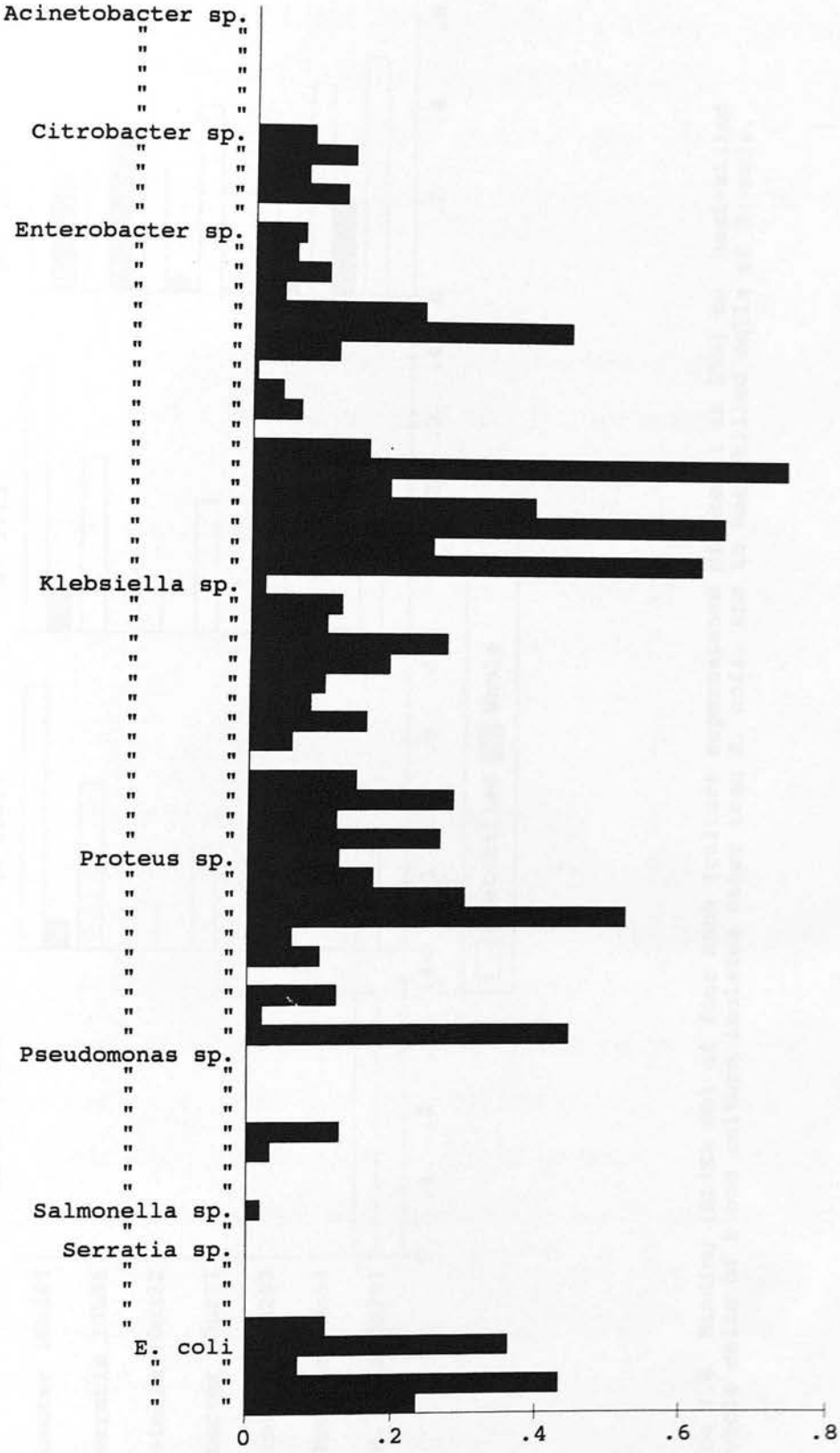


Figure 7.3 Binding (ELISA OD) of MAb WN1 16.1 (culture supernatant diluted 1 in 250) to heat-killed cells of 69 blood culture isolates of bacteria other than *E. coli* and four faecal isolates of *E. coli*.

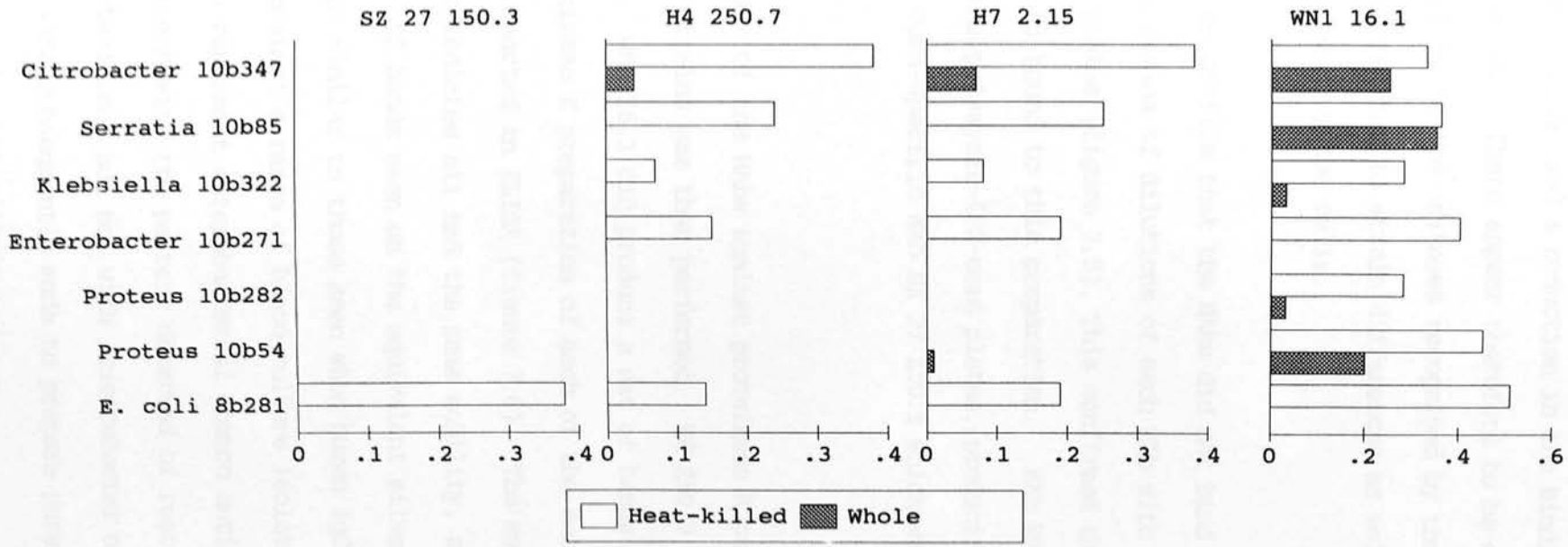


Figure 7.4 Binding (ELISA OD) of four MAbs (culture supernatants diluted 1 in 250) to heat-killed and whole cells of blood culture isolates other than *E. coli*, and to heat-killed cells of *E. coli*.

250.7 and H7 2.15, and a reduction in the binding of WN1 16.1 to some of the strains. There appear therefore to be differences in the accessibility of the epitopes recognised by these MAbs, and these seem to be related to strain differences as well as to differences caused by heating the cells.

In order to confirm that the MAbs did not bind LPS-core I next reacted a series of dilutions of each MAb with polymyxin-LPS-core cocktail plates (figure 7.5). This confirmed that neither H4 250.7 nor H7 2.15 bound to this preparation. MAb WN1 16.1 did however bind to the polymyxin-LPS-core plates, producing higher OD values than the core-specific MAb SZ 27 150.3 which was used as a positive control.

Immunoblots of the MAbs against proteinase K preparations of the selected strains was then performed. H4 250.7 and H7 2.15 produced no bands. WN1 16.1 did produce a set of bands in the immunoblot with the proteinase K preparation of each of the selected isolates with which it reacted in ELISA (figure 7.6). The sets of bands from the different isolates all had the same mobility, and did not coincide with the LPS bands seen on the equivalent silver-stained gel. These bands were similar to those seen when human IgG preparations were blotted against a range of blood-culture isolates, and were thought likely to represent enterobacterial common antigen (ECA). This would be consistent with the pattern observed of reactivity with many Enterobacteriaceae but not with *Acinetobacter* or *Pseudomonas*. Attempts were subsequently made to prepare pure ECA [146] to test this hypothesis, but without success.

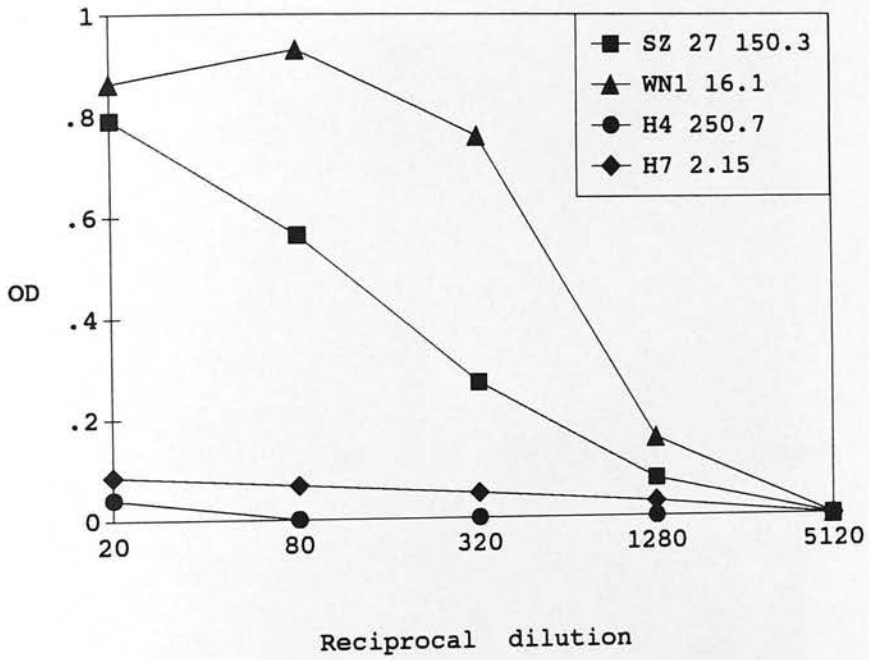


Figure 7.5 Binding (ELISA OD) of 4 MAbs to polymyxin-LPS-core cocktail.

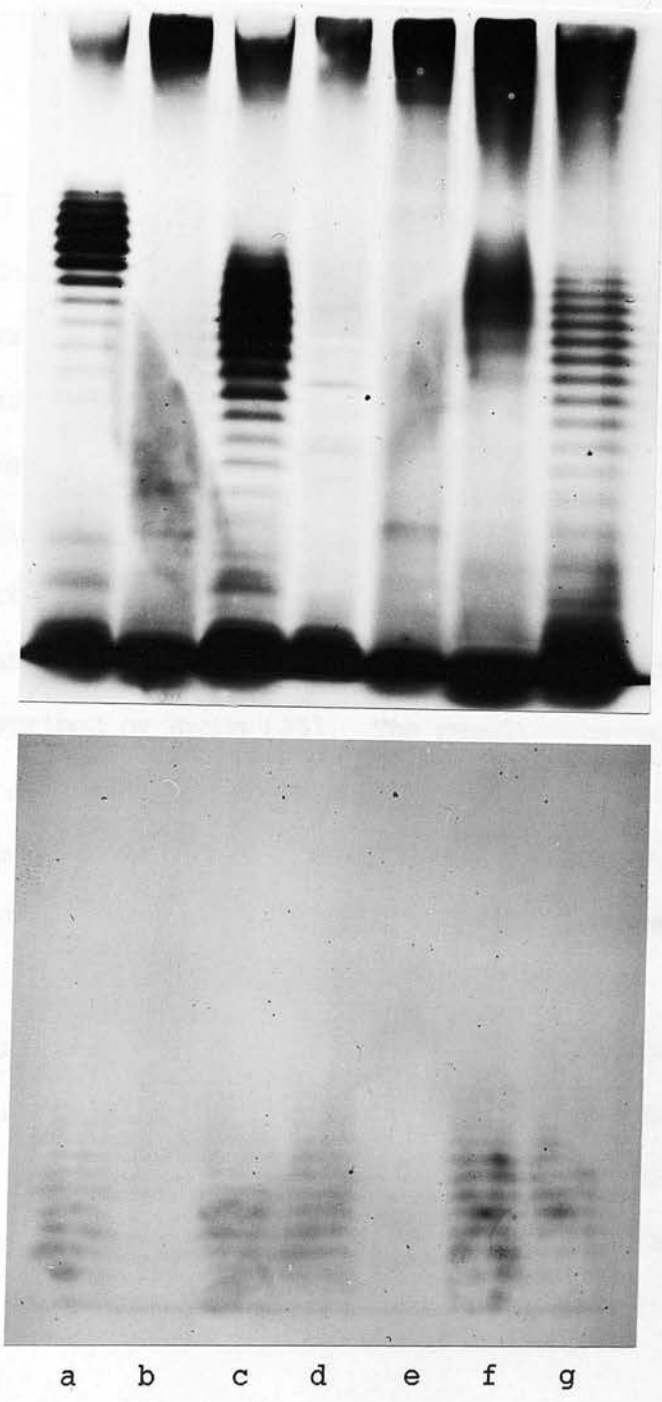


Figure 7.6 Silver stain (top) and immunoblot with MAb WN1 16.1 (bottom) of polyacrylamide gel of proteinase K digest of Enterobacteriaceae. Lanes a = *E. coli* 018 (10b281); b = *Proteus* sp. (10b54); c = *Serratia* sp. (10b85); d = *Enterobacter* sp. (10b271); e = *Proteus* sp. (10b282); f *Klebsiella* sp. (10b322); g = *Citrobacter* sp. (10b347).

Immunoblots were then performed with outer membrane protein (OMP) preparations from four of the selected strains. The initial blot was performed using 10% polyacrylamide in the separating gel. MAb WN1 16.1 gave a negative result in this experiment.

H4 250.7 and H7 2.15 reacted with a narrow band at the gel front in the tracks containing OMP from the *E. coli*, *Citrobacter* and *Serratia* strains, but produced no band in the track containing OMP from the *Proteus* strain. This was consistent with the pattern of reactivity observed with heat-killed bacteria in ELISA. The bands seen in the immunoblot corresponded to bands seen on the Coomassie-blue stained preparation. This suggested that the MAbs were reacting with an OMP of low molecular weight, possibly the lipoprotein described by Braun [35]. The result obtained with H4 250.7 is shown in figure 7.7. It is of note that the Coomassie-blue stained gel shows an equivalent low molecular weight OMP in the *Proteus* preparation, although this is not seen in the immunoblot.

In an attempt to resolve the low molecular weight protein more precisely the immunoblots were repeated using 14% acrylamide in the separating gel, as shown in figure 7.8. Under these conditions the narrow band seen in the Coomassie-blue stained gel was replaced by a broader band. MAbs H4 250.7 and H7 2.15 reacted with all of this broad band.

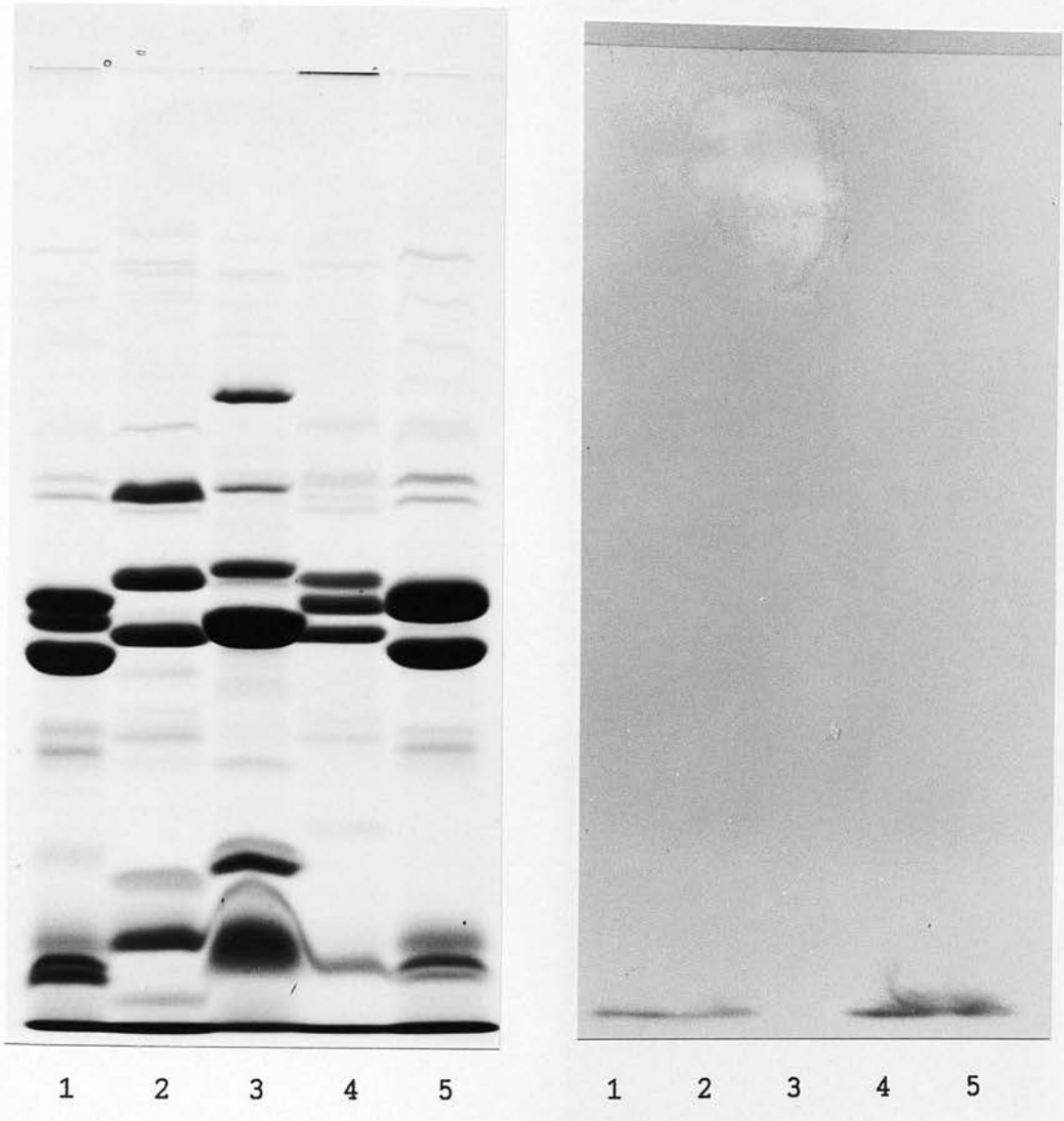


Figure 7.7 Immunoblot of H4 250.7 against outer-membrane protein preparations separated on a 10% polyacrylamide gel. Left: Coomassie blue stained polyacrylamide gel. Right: Immunoblot. Lanes 1 & 5 = *E. coli* O18 (8b281); 2 = *Serratia* sp. (10b85); 3 = *Proteus* sp. (10b282); 4 = *Citrobacter* sp. (10b347).

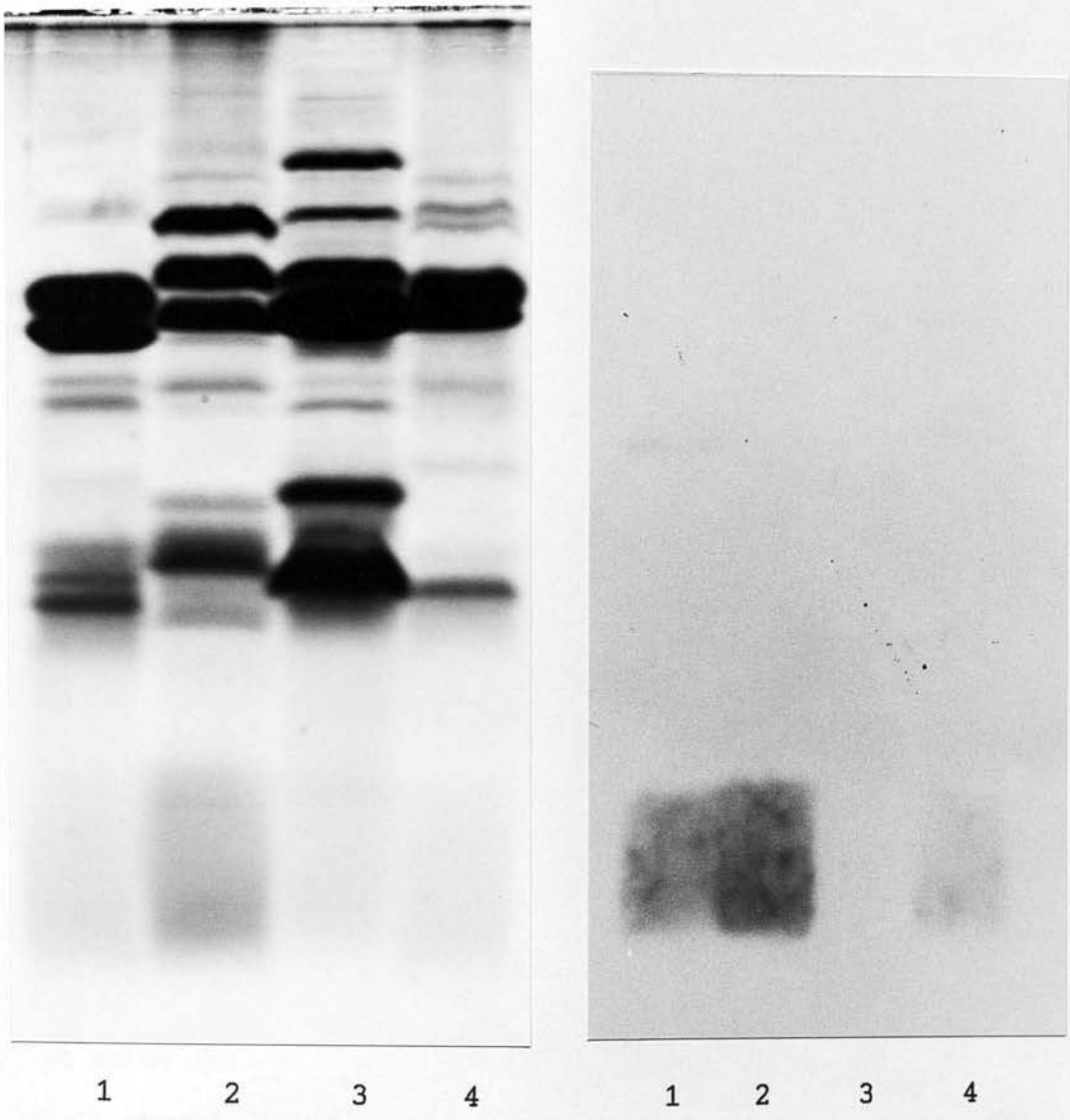


Figure 7.8 Immunoblot of H4 250.7 against outer-membrane protein preparations separated on a 14% polyacrylamide gel. Left: Coomassie blue stained polyacrylamide gel. Right: Immunoblot. Lanes 1 = *E. coli* O18 (8b281); 2 = *Serratia* sp. (10b85); 3 = *Proteus* sp. (10b282); 4 = *Citrobacter* sp. (10b347).

HA-1A (Centoxin)

A small supply of HA-1A was obtained by salvaging material from "empty" bottles from the pharmacy at the Royal Infirmary of Edinburgh after this MAb became commercially available. These bottles typically contained 100 µl of a 5 mg/ml solution of HA-1A. Bottles were stored at 4°C. The first sample obtained was used for an ELISA experiment using heat-killed *E. coli* R3 as antigen. As seen in figure 7.9 the MAb bound to the *E. coli* but not to control wells which had been coated with albumin. Similar results for binding to polymyxin-LPS-core were obtained with this same sample of HA-1A by Dr GR Barclay (not shown).

From the data in figure 7.9, 20µg/ml was selected as a reasonable concentration of HA-1A to use to assess its ability to bind to other bacterial preparations in ELISA, and this concentration was used to assess the binding of HA-1A to a range of other organisms in ELISA. Initially a range of Gram-negative organisms was used. Figure 7.10 shows the results expressed net of the blank reading obtained from wells with no HA-1A. In this assay there was a measurable amount of non-specific binding of the MAb to wells which contained no antigen but which had been post-coated compared to wells which had no HA-1A. HA-1A showed some degree of binding to all of the Gram-negatives tested. The binding was greatest with heat-killed cells rather than whole cells, and in some cases the binding to whole cells was little higher than that obtained with no added bacterium.

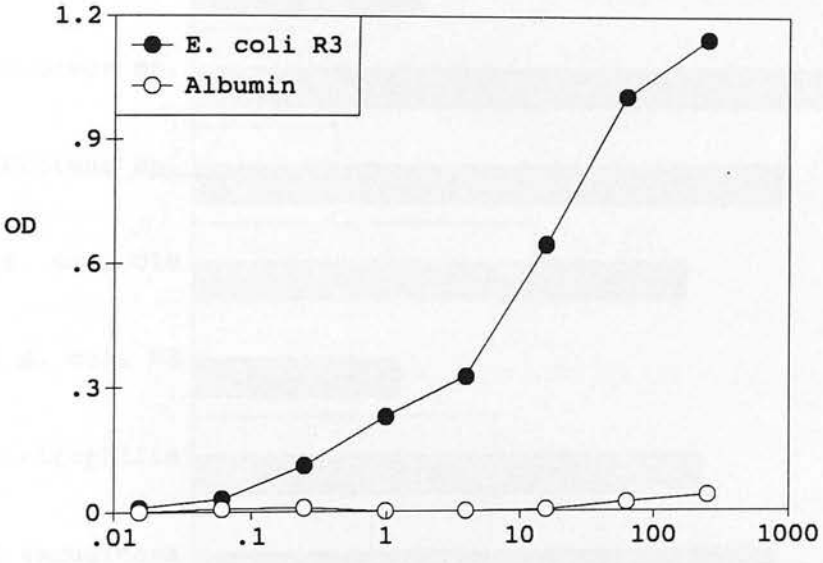


Figure 7.9 Binding (ELISA OD) of Centoxin to heat-killed *E coli* R3 post-coated with albumin and to albumin alone.

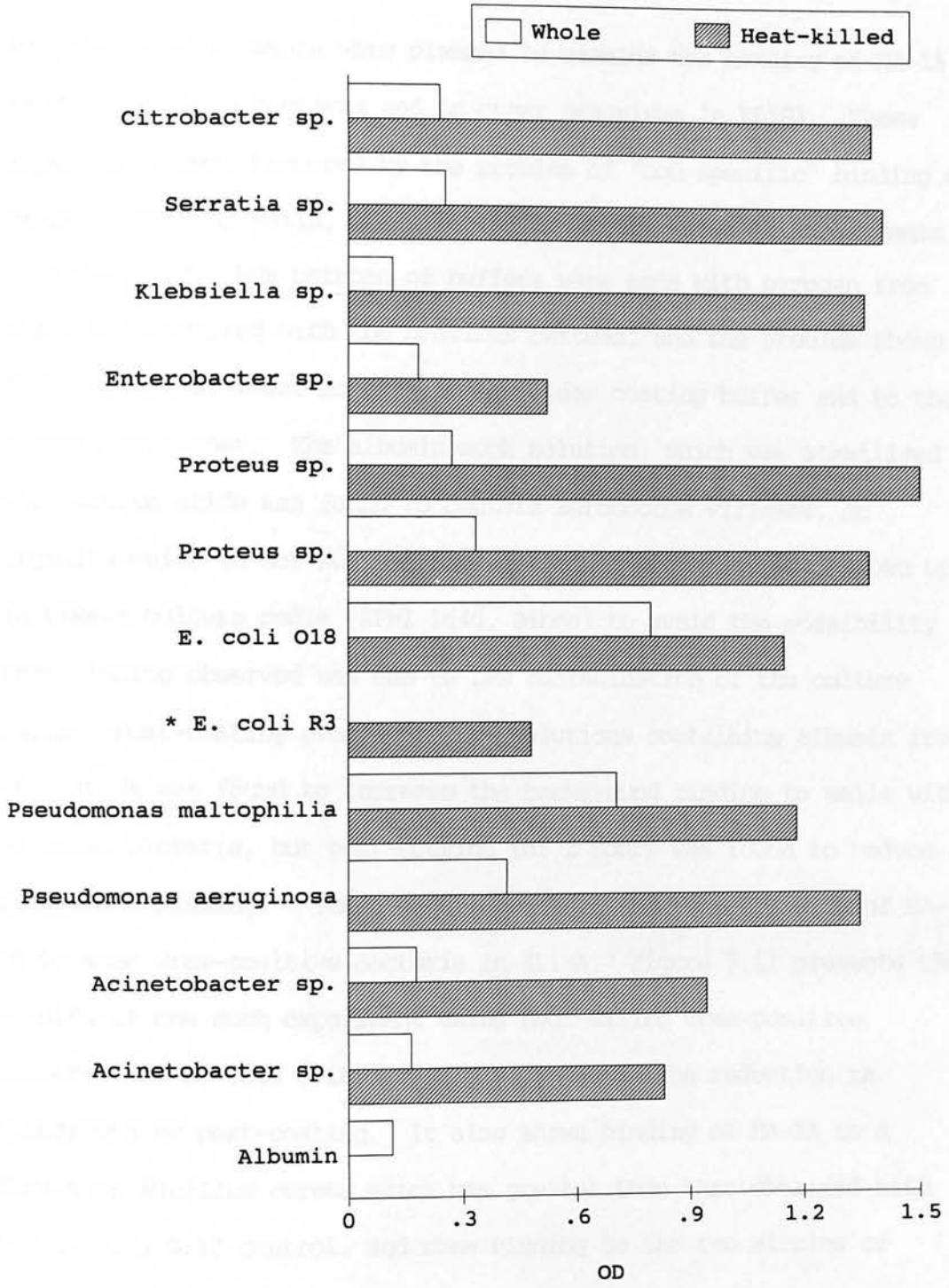
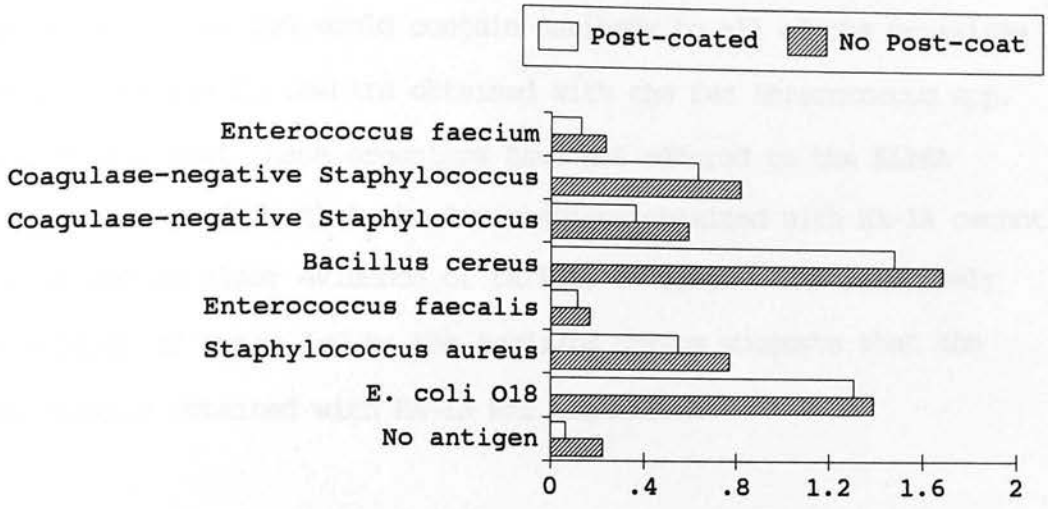
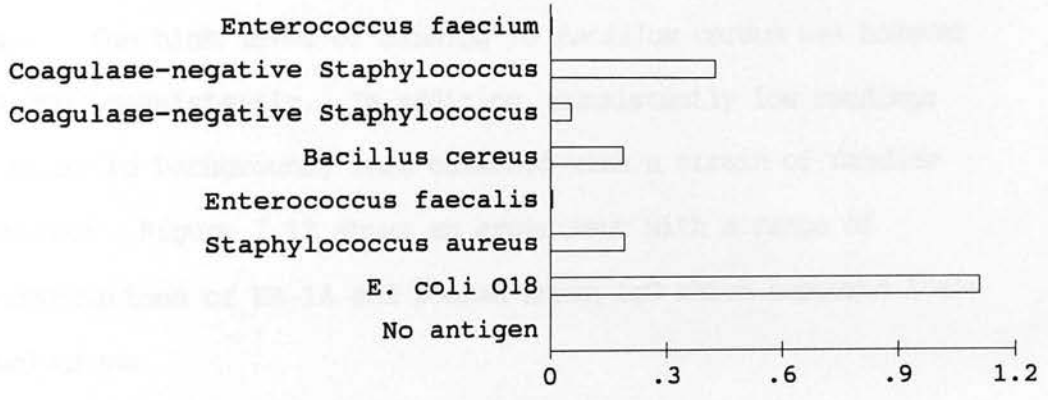


Figure 7.10 Binding (ELISA OD) of HA-1A (20 μg/ml) to whole and heat-killed Gram-negative bacteria post-coated with albumin and to albumin alone.
* In the case of *E. coli* R3 only heat-killed cells were used.

Subsequent experiments were planned to examine the binding of HA-1A to Gram-positive bacteria and to other organisms in ELISA. These experiments were hampered by the problem of "non-specific" binding of HA-1A to control wells, which seemed to become worse as experiments were repeated. New batches of buffers were made with pyrogen free water and compared with the previous batches, and the problem shown to be relate at least in part to the older coating buffer and to the albumin post-coat. The albumin sock solution, which was stabilised with sodium azide was found to contain *Aerococcus viridans*, an organism which is not susceptible to azide. Organisms were grown up in tissue culture media (RPMI 1640, Gibco) to avoid the possibility that binding observed was due to LPS contamination of the culture media. Post-coating overnight with solutions containing albumin from this stock was found to increase the background binding to wells with no added bacteria, but post-coating for 2 hours was found to reduce background binding. These experiments did show some binding of HA-1A to some Gram-positive bacteria in ELISA. Figure 7.11 presents the results of one such experiment using heat-killed Gram-positive bacteria and *E. coli* 0:18. Figure 7.11a shows the reduction in background by post-coating. It also shows binding of HA-1A to a strain of *Bacillus cereus* which was greater than that obtained with the *E. coli* 0:18 control, and some binding to the two strains of coagulase-negative staphylococcus. No binding of conjugate was obtained in the absence of HA-1A, confirming that the binding observed is not due to direct binding of the conjugate to the organisms. Whole *Staphylococcus aureus* cells did bind the conjugate under these conditions (not shown). Figure 7.11b shows results obtained in a parallel experiment using pooled human IgG. This



a) HA-1A



b) Pooled human IgG

Figure 7.11 Binding (ELISA OD) of a) HA-1A (20 μg/ml), and b) pooled human IgG (100 μg/ml), to heat-killed *E. coli* O18 and heat-killed Gram-positive bacteria.

experiment was intended as a positive control, since it was expected that pooled human IgG would contain antibody to all of the organisms tested. The low OD results obtained with the two *Enterococcus* spp. may indicate that these organisms have not adhered to the ELISA wells, and therefore that the low readings obtained with HA-1A cannot be regarded as clear evidence of failure to bind. The relatively low binding of human IgG to the *Bacillus cereus* suggests that the high binding obtained with HA-1A was significant.

Further ELISA experiments were carried out with other organisms, but interpretation was hampered by high background readings in many cases. The high level of binding to *Bacillus cereus* was however observed consistently. In addition, consistently low readings (similar to background) were obtained with a strain of *Candida albicans*. Figure 7.12 shows an experiment with a range of concentrations of HA-1A and pooled human IgG which supports these conclusions.

Immunoblotting of HA-1A against LPS preparations was also attempted, with largely negative results. An initial experiment used a single wide blot of a proteinase K preparation of *E. coli* 0:18 LPS cut into strips. HA-1A at concentrations of 250µg/ml, 50µg/ml, and 17µg/ml of HA-1A were used, and produced a diffuse darkening of the blot which was not related to the LPS bands, but which did decrease in intensity with decreasing HA-1A concentration. This indicated non-specific binding of the HA-1A to the blot, and also indicated that the peroxidase anti-human-IgM conjugate was functioning.

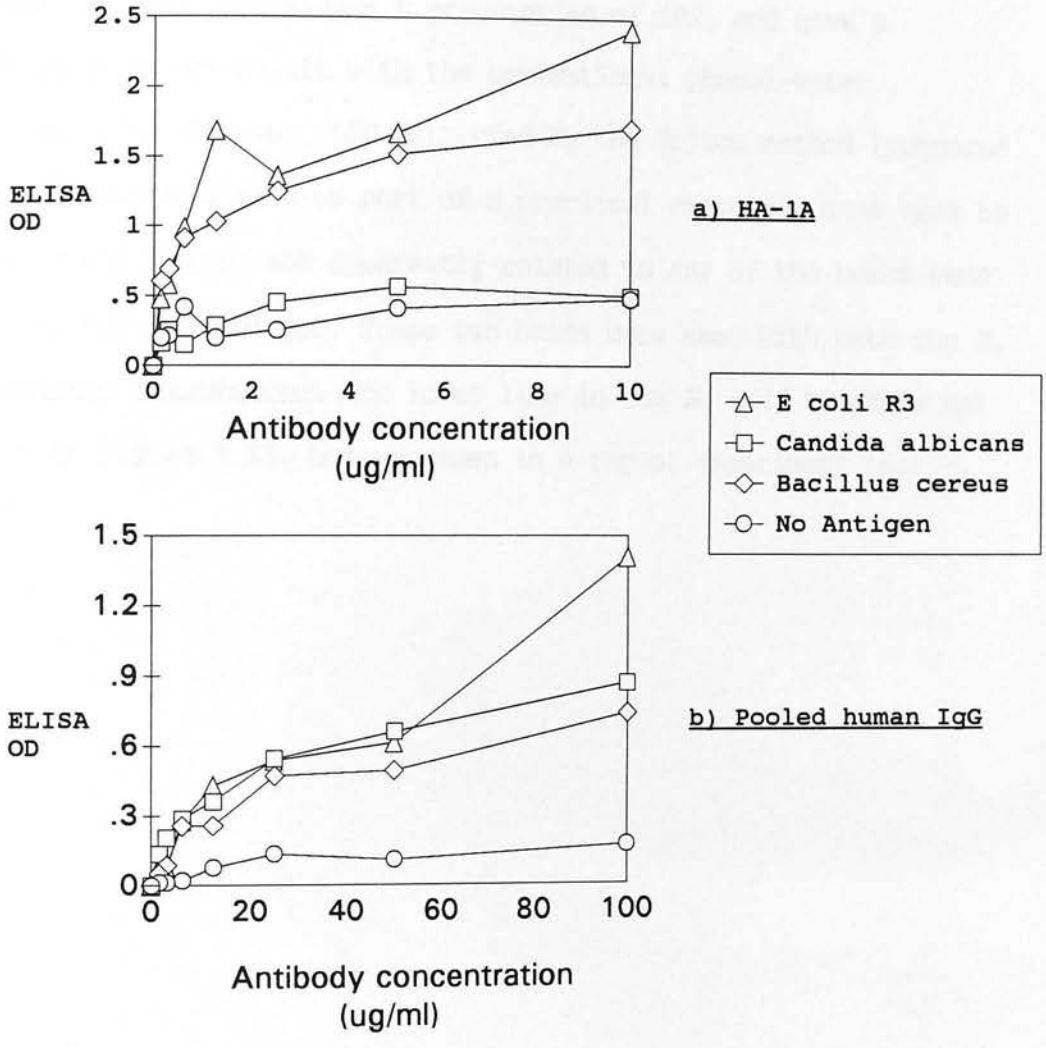


Figure 7.12 Binding (ELISA OD) of a) HA-1A, and b) pooled human IgG, to heat-killed microorganisms.

A further immunoblot was carried out using LPS from *E. coli* 0:18 and from a *Citrobacter* sp. prepared by three different methods (figure 7.13). This experiment confirmed the essentially negative result obtained with the proteinase K preparation of LPS, and gave a similarly negative result with the conventional phenol-water extracted LPS. However, LPS extracted by the Triton method (prepared by two honours students as part of a practical exercise) gave rise to two bands which were not apparently related to any of the bands seen on the silver-stained gel. These two bands were seen with both the *E. coli* and the *Citrobacter*. The lower line in the *E. coli* track is not visible in figure 7.13, but was seen in a repeat experiment (not shown).

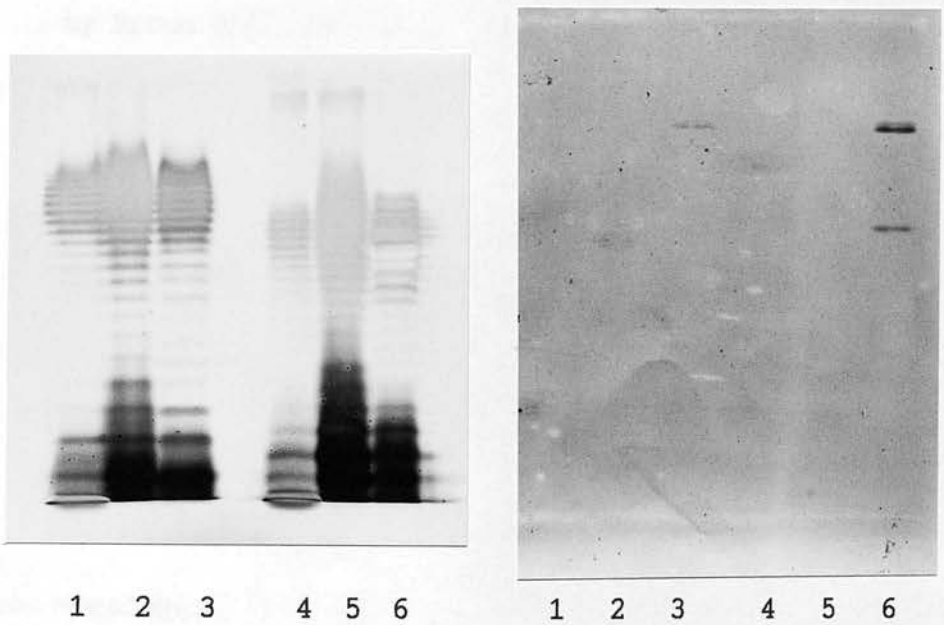


Figure 7.13 Immunoblot of HA-1A (25 µg/ml) against LPS, prepared by three different methods, from *E. coli* 8b281 (lanes 1,2,3) and *Citrobacter* sp. 12b274 (lanes 4,5,6). Left: Silver-stained polyacrylamide gel; Right: Immunoblot. Lanes 1 & 4 = proteinase K digest, 2 & 5 = phenol-water extract, 3 & 6 = Triton extract.

DISCUSSION

Three distinct types of MAb are described in this chapter.

MAb H4 250.7 and H7 2.15

MAb H4 250.7 and H7 2.15 bind to an outer membrane protein of *E. coli* and some other Enterobacteriaceae, with the notable exception of *Proteus* spp. This protein is probably the lipoprotein described in *E. coli* by Braun [35] which has a molecular weight of 7300. Lipoproteins from *E. coli*, *Proteus mirabilis*, *Morganella morganii*, *Erwinia amylovora* and *Serratia marcescens* have been sequenced [263]. Of the 58 amino acids in *E. coli* free lipoprotein, 29 are the same in all these organisms, and the rest show some variation. The greatest differences are between *E. coli* and *Proteus mirabilis*, which have only 33 amino acids in common. *E. coli* and *Serratia marcescens*, in contrast, have 54 of the 58 amino acids in common.

Earlier serological studies (reviewed by Braun [35]) have shown that antibodies to *E. coli* lipoprotein do not react with *Proteus* but do react with some other Enterobacteriaceae. Interpretation of these serological studies is however complicated by the different methods used to prepare lipoprotein. "Lysozyme-lipoprotein" was prepared by lysozyme, which left two repeating units of peptidoglycan attached to the C-terminal of lipoprotein. Alternatively "sugar-free lipoprotein" was prepared with trypsin, but this lacked the C-terminal lysine residue. Antisera to these preparations of lipoprotein from *E. coli* did not cross-react with one another,

indicating that the C-terminal end was the dominant epitope of lipoprotein. These antisera also showed differing degrees of cross-reactivity with equivalent lipoprotein preparations from other Enterobacteriaceae.

The lipoprotein present in the OMP preparations described in this thesis is expected to be the "free" form which can be extracted without enzyme treatment. An antiserum to free lipoprotein from *E. coli* was shown by Halegoua et al [91] to react with lipoprotein from *Salmonella* and *Serratia* but not *Proteus* in precipitation experiments. However, Henricksen et al [92] found that antiserum to *E. coli* free lipoprotein did react with *Proteus* lipoprotein in immunoblots. It is likely that the cross-reactive antiserum described by Henricksen et al [92] included antibodies to epitopes shared by *E. coli* and *Proteus*. It is clear from the sequence data that such regions do exist.

The finding of two MAbs which react with lipoproteins from several enterobacterial genera other than *Proteus* is therefore consistent with the available data on lipoproteins.

The two MAbs show such similar patterns of reactivity with the range of bacteria tested that they are likely to share the same epitope. In view of the difference in specificity between MAb H4 250.7 from batch 1 (*E. coli* R3 specific) and MAb H4 250.7 from batch 2 described here it is clear that some mistake in labelling has occurred. It is possible that one clone has been given two different labels and therefore that the two MAbs may be identical.

These MAbs might be of value in studies of the structure of Gram-negative bacterial cell envelopes. The epitope recognised appears to be relatively inaccessible in standard broth-grown smooth organisms. It may be more accessible in rough strains [35] or in bacteria grown under non-standard conditions.

MAb WN1 16.1

This MAb is thought to react with ECA, for the following reasons: a) it reacts with Enterobacteriaceae, but not with other Gram-negative bacteria; b) it reacts in immunoblots with proteinase K-resistant material which is not detectable on silver stained gels; c) the bands seen in the immunoblot are identical between different enterobacterial species; and d) the bands are similar to those found by others in immunoblots of ECA [121,183]. Confirmation of the specificity of this MAb would require purified ECA with which to test its reactivity.

The binding of this MAb to polymyxin-LPS-core cocktail ELISA was unexpected as it had been found not to bind to a range of smooth LPS in ELISA (data not shown). A likely explanation for this result is the presence of ECA as an unexpected component of the LPS-core cocktail. This is most likely to be in the form of ECA_{LPS} [121], which consists of ECA covalently bound to LPS-core. ECA_{LPS} is found in the LPS-core fraction when the aqueous phenol, chloroform, petroleum ether method used to prepare rough LPS. ECA_{LPS} is found in some enterobacterial rough mutants, in particular *E. coli* K12 ([120],

cited by [121]) which is a component of the polymyxin-LPS-core cocktail. This observation is important for the interpretation of antibody measurements made with polymyxin-LPS-core cocktail ELISA plates, since antibodies to ECA may be detected.

Other anti-ECA MAbs have been described, and a possible application of such MAbs is the detection of Enterobacteriaceae in drinking water [98,129,183]. It might also be possible to use these MAbs in the study of Gram-negative bacterial cell envelopes under different conditions, as described above. WN1 16.1 could also be used to detect ECA contamination of rough LPS preparations.

HA-1A

A surprising limited amount of information is available on the *in-vitro* activity of HA-1A. In the original description of this MAb [230] culture supernatants with an antibody content of 8-20 µg/ml reacted with a variety of Gram-negative bacteria in ELISA at a dilution of between 1 in 16 and 1 in 128 for most organisms tested. The minimum MAb concentration showing activity in ELISA must therefore be 0.06-1.25 µg/ml. Binding to lipid A in ELISA was reported in the product monograph [3] to be just above background at a similar range of concentrations. The OD rose steeply with rising MAb concentration up to 20µg/ml. Similar findings are reported in less detail in two abstracts [20,22]. Lipid A was said to inhibit competitively this binding in the original report [230]. Binding was also reported to LPS in immunoblots, and to chitin which is a polymer of N-acetylglucosamine. No negative control studies, such as failure

of HA-1A to bind to Gram-positive bacteria or other biological materials, were reported. An affinity constant for Re LPS of 10^7 M^{-1} was claimed [265].

Baumgartner et al [16] have challenged the specificity of the MAb for lipid A, reporting that HA-1A binds to a wide range of Gram-positive bacteria, to fungi, and to lipids unrelated to lipid A. This group reported an affinity constant for lipid A of 10^4 M^{-1}, and suggested that the binding observed represented non-specific interactions with hydrophobic substances.

The results reported in this chapter support the idea that HA-1A is not specific for lipid A, but rather that it interacts with a variety of substances. The concentrations used in ELISA experiments was similar to those discussed above, suggesting that the binding observed was not simply a result of using excessive amounts of MAb. These experiments are unsatisfactory however, because of the considerable and variable background binding that was observed. This could be taken as evidence of non-specific binding. The background binding might however be due to the presence of contaminating LPS somewhere in the system. Efforts were made to avoid LPS contamination by using endotoxin-free water, and by growing organisms up in tissue-culture media, but the possibility cannot be absolutely excluded. Alternatively, the quality of the HA-1A preparations used might be questioned because of the way they were obtained. However, the preparations tested here are of the same quality as those given to patients.

The reaction of HA-1A with two bands of uncertain nature in LPS prepared by the relatively new Triton method serves to confirm that the immunoblotting reagents were working. The nature of these bands is obscure, but it appears that they are something which bind HA-1A more strongly than does LPS.

The lack of a clear definition of the *in vitro* activity of HA-1A was one of the criticisms made of this MAb following the clinical trial [249]. If the MAb is really not lipid A specific then there is no rational basis for its use. It is clearly of enormous importance to potential users of HA-1A to establish this basic point. It may not however be in the interests of Centocor to pursue these studies.

CHAPTER 8

CONCLUSIONS

Seeing is believing, but, if you're not careful,
believing is seeing

JH Collins

The concept of cross-reactive antibodies to LPS as a mechanism of natural immunity began with a paper by Chedid et al in 1968 [40]. The protective element described was however heat-labile and seemed to require an "enzymatic process capable of ... unmasking R antigenic structures [of bacteria]" for its activity, which was bactericidal. The concept has moved on considerably since then but still suffers from the lack of a clear theoretical basis defining the epitope to which such antibodies should be directed, and the mechanism by which they might work.

Antibodies to complete LPS-core, or to Rc core which may share the cross-reactive epitopes [10] may occur naturally, and may well have a protective role [188]. Measuring such antibodies is a problem, however, and no standard method has emerged. The difficulties include the detection of highly specific antibodies, for example to *E. coli* J5 LPS [14,17], though this type of antibody may only be found after immunisation.

The main method used to detect naturally-occurring cross-reactive antibodies in this thesis has been polymyxin-LPS-core cocktail ELISA. This method is certainly open to the criticism that it may be detecting a number of specific antibodies to LPS-core (antibodies equivalent to the J5-specific antibodies) rather than truly cross-reactive antibodies. This may not be important when the assay is

used for its original purpose of selecting donor sera for the manufacture of an "anti-LPS" immune globulin, since infusion of a range of specific antibodies may have a useful therapeutic effect. It would be important however when using the assay to show the existence of truly cross-reactive antibodies. The method has two other problems: the presence of polymyxin and the possible presence of antigens than LPS-core, notably ECA. Polymyxin has been shown to compete with some cross-reactive MAbs in binding to the lipid A-inner core region [7,163,189], and therefore the assay will probably fail to detect equivalent naturally-occurring antibodies. ECA seems likely to be present in the LPS-core cocktail as a component of the *E. coli* K12 component ([120], cited by [121]), and was apparently detected by the ECA-specific MAb WN1 16.1. Antibodies to ECA do occur naturally, but are not apparently protective in Gram-negative bacteraemia [150].

Despite these limitations, the polymyxin-LPS-core assay was suitable for the purposes for which it was used in this thesis. In the studies of typhoid vaccinees the original question raised was whether typhoid vaccination might have induced the production of antibodies which had been detected by the polymyxin-LPS-core ELISA. If more positive results had been obtained it would have been important to investigate the antibodies by other methods, but the generally negative results obtained were sufficient to answer the question posed.

The studies on antibodies in urine serve as an example of the danger

of seeing what you believe to be present. McKenzie and Young [156] looked for antibodies to bacteria causing urinary infection, and found them. I looked for antibodies to LPS-core (as defined by the polymyxin-LPS-core cocktail ELISA) and found them. The likelihood is however that the antigen used was irrelevant to these studies, and that any antigen to which humans are commonly exposed would do. Similar considerations may relate to other studies of naturally occurring antibodies. Thus the finding that antibodies to *E. coli* J5 [188] or *Salmonella* Re [152] LPS are associated with improved outcome from bacteraemia may be explained simply by cross-protective activity of the antibody measured, but it is possible that the measured antibody was associated with some other defensive mechanism, such as the presence of a variety of specific protective antibodies.

There has been growing interest in monoclonal antibodies to LPS in recent years. In the first 50 weeks of 1992, the Science Citation Index listed 127 papers with "monoclonal" and "lipopolysaccharide(s)" in the title or as key words. With the growth of interest in monoclonal antibodies to LPS has come increasing sophistication in their assessment. The original description of HA-1A [230], for example, would surely not be acceptable to a peer-reviewed journal now, only eight years later. HA-1A is, in my opinion, another example of the danger of seeing what was believed to be present.

The MAbs described in this thesis have not been subjected to the most detailed of the analyses now available. The epitopes which they recognise could be characterised by competition studies with synthetic partial structures. It would be impractical to do this for

all the MAbs mentioned in this thesis, but it would be of interest to study some, particularly those core-type-specific MAbs, and the "all-*E. coli*" MAbs. Such studies would be a major undertaking, and would ideally include comparisons with core-type-specific MAbs from other sources [133,170,235,245] and determination of affinity constants for the defined epitopes. The ability of the MAbs to bind to whole bacteria, as distinct from isolated LPS, has not been systematically studied, but it is apparent from other work that this question depends crucially on the way in which bacteria are grown [153], and cannot therefore lead to practical conclusions until the phenotype of bacteria causing infection is better understood. The effect of the MAbs studied here on the biological activities of LPS have not been discussed. Such studies are being undertaken by others, and show promise [58], but it is not possible to draw simple conclusions about their possible benefit in human infections.

Useful conclusions can be drawn from the studies in this thesis however. Many of the MAbs produced have been found to react with*E. coli* and *Salmonella* LPS, but not with LPS from other Gram-negatives. The nature of the MAbs obtained was presumably determined by the predominance of *E. coli* and *Salmonella* LPSs in immunisation and selection procedures.

MAbs have been described which react with complete LPS molecules from nearly all *E. coli* and *Salmonella* strains tested. This sort of relatively specific antibody seems likely to be of benefit in illness caused by *E. coli* or *Salmonella* LPS, assuming that the binding of antibody results in either reduced toxicity or enhanced clearance of

LPS. In contrast, MAbs capable of binding only to free lipid A [122] or incomplete core structures [130] seem unlikely to of benefit [28]. Although lipid A and incomplete core structures are more conserved among Gram-negative bacterial species they are probably not found in infections, except as part of complete LPS-core or O-substituted LPS molecules. To guide the use of relatively specific MAbs (such as the "all-*E. coli*" MAbs) it would be necessary to have a more precise knowledge of the types of LPS involved in human disease. It might be, for example, that an "all-*E. coli*" MAb together with an equivalent anti-*Pseudomonas aeruginosa* MAb [167] and an anti-*Klebsiella* and *Enterobacter* MAb would prove to be a powerful combination. Alternatively, it may be that an antibody to *Bacteroides* LPS is required, or it may be that an "all-*E. coli*" MAb will react with enough clinically significant LPS to be useful by itself. It appears to me unrealistic to expect to find a single antibody which will bind to complete LPS molecules from all these species.

The core-type-specific MAbs seem unlikely to be of therapeutic use simply because of their failure to react with a sufficiently wide group of organisms. Some do however show protective activity in some animal models (unpublished data) and might be useful in improving our understanding of these animal models. They could for example be used to study the relationship between suppression of TNF production and protection in whole animal systems.

The anti-ECA and anti-lipoprotein MAbs seem unlikely to be of therapeutic value either, because they do not recognise the

appropriate toxic molecule. There is also evidence that antibodies to ECA are not protective in humans [150] or in animal models [149]. Negative results in such experiments by one investigator do not however absolutely rule out the possibility of beneficial effects [264]. Anti-ECA or anti-lipoprotein antibodies might play some part in the protective effects of immunisation with rough bacteria [37] or transfer of sera from immunised donors [106,267], and might go some way to explain the failure to find the expected anti-LPS antibodies in sera from these studies [14], and the observation that these sera are more protective against live infection than against endotoxin challenge [264]. Immunisation with rough bacteria does lead to the production of antibody to lipoprotein ([144] cited by [35]) and ECA [121], while immunisation with smooth bacteria does not. It would therefore be interesting to investigate the bactericidal activity of these MAbs.

Cross-reactive antibodies to LPS still have considerable potential for therapeutic use, although the initial wave of enthusiasm may have passed. The continuing challenge is to decide which antibodies and which investigations deserve an investment of time and money, and to achieve the scientific imperative of openness and objectivity despite the commercial pressures for secrecy and product promotion.

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PUBLICATIONS

Gibb AP & Edmond DM (1992). Urinary IgG antibody against mixed heat-killed coliform antigen and lipopolysaccharide core antigen. J Clin Pathol **45**:161-164.

Gibb AP, Barclay GR, Poxton IR & di Padova F (1992). Frequencies of lipopolysaccharide core types among clinical isolates of *Escherichia coli* defined with monoclonal antibodies. J Infect Dis **166**:1051-1057

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Urinary IgG antibody against mixed heat-killed coliform antigen and lipopolysaccharide core antigen

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Abstract

Aims: **To determine whether antibody to lipopolysaccharide-core(LPS-core) antigen is an important component of the antibody, detected by mixed heat-killed coliform antigen, in urine from patients with suspected urinary tract infection.**

Methods: **LPS-core antigen and mixed heat-killed coliform antigen were used in an enzyme linked immunosorbent assay (ELISA) to measure IgG antibody in mid-stream urine samples. Seventy two samples from students attending their general practitioner with symptoms suggestive of urinary tract infection, six samples from which a Gram positive organism was isolated, and 16 asymptomatic controls were tested. Plates coated with LPS-core antigen were also used to absorb out the antibody detected by the mixed heat-killed coliform antigen.**

Results: **Antibody to either antigen was associated with a positive culture, but neither was a useful predictor of a positive culture. There was a significant correlation between the results of the two assays ($r = 0{\cdot}7633$; $p < 0{\cdot}001$), and absorption with LPS-core antigen did reduce the level of antibody to the mixed heat-killed coliform antigen. Antibody to both preparations was found in patients with Gram positive urinary tract infection.**

Conclusions: **Antibody to LPS-core antigen forms a substantial part of the antibody detected by mixed heat-killed coliform ELISA. The antibodies detected by these assays are probably the result of non-specific leakage of antibody into the urine, rather than a specific immune response.**

Studies of antibacterial antibody in urine during urinary tract infection have mainly focused on specific responses to organisms isolated from individual patients,[1–4] and have been mainly directed towards distinguishing between pyelonephritis and cystitis.[1 3] An alternative approach has been to look for urinary antibody to a wide range of likely pathogens, and to use this as a tool for the primary diagnosis of urinary tract infection.[5 6] A test based on this concept (Uristat, Shield Diagnostics, Dundee) has been marketed in the United Kingdom. This approach to the diagnosis of urinary tract infection has the theoretical advantage that urinary antibody would not be prone to the problems of contamination and overgrowth associated with quantitative bacterial culture, and would be more stable than pus cells. It has the disadvantage, however, that there is a dearth of basic information about the clinical importance of this kind of antibody in urinary tract infection.

The aim of this study was to investigate the nature and clinical relevance of the antibody detected by the mixed heat-killed coliform ELISA (MHCE) described by Mackenzie and Young.[5] It was decided to concentrate on IgG because this was the main antibody class that had been detected in that assay. Secretory IgA was considered but rejected on the grounds that it has not been found consistently in urine in urinary tract infection.[4 5] The core region of LPS (LPS-core) was thought likely to be an important antigen in the assay for two reasons. First, LPS-core is a heat stable antigen which is present in all Gram negative bacteria. It would thus be present in the MHCE antigen preparation used. Second, LPS-core antibody is found frequently in serum from the normal human populations,[7] confirming that this antibody is part of the human immunological repertoire. A urinary antibody response to urinary tract infection might therefore be expected to include antibody to LPS-core antigen, and this antibody would be detected by the MHCE assay.

Methods

Seventy two urine specimens were collected from students with suspected urinary tract infection attending general practitioners at the University of Edinburgh Students' Health Centre. None of these patients was thought to have pyelonephritis. One patient who was known to be taking antibiotics was excluded. Most were subsequently treated with antibiotics, and none returned because of persisting symptoms. These specimens were collected under the supervision of an experienced nurse and were then brought directly to the diagnostic laboratory by the patient. Urine specimens from patients with Gram positive urinary infection were selected after culture from among all specimens sent to the diagnostic laboratory by general practitioners. Control samples were obtained from 16 asymptomatic students.

Microscopy was performed using an inverted microscope. Ten pus cells per field

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(equivalent to 20 per mm^3) was scored positive. Cultures (standard loop) containing $>10^5$ colony forming units (cfu) ml of a single bacterial species were considered positive, and specimens containing 10^4–10^5 cfu/ml were reported as doubtful.

Urine samples were centrifuged at 4000 × *g* for 10 minutes and the supernatant stored in 1 ml volumes at −20° C before analysis for antibody.

ELISA PLATES

For MHCE assay, plates were coated with a mixture of six coliform organisms (four *E coli*, one *Klebsiella*, one *Proteus*) isolated from urine specimens. The coating suspension was prepared as follows: each organism was grown overnight in nutrient broth at 37° C, washed, and resuspended in phosphate buffered saline (PBS). The concentration was adjusted so that a 1 in 10 dilution had an extinction level of 0·25 at 540 nm. The suspension was then heated to 100°C for 30 minutes. The six preparations were mixed together in equal parts and the mixture diluted 1 in 10 with 0·05M carbonate-bicarbonate coating buffer (pH 9·6). Nunclon polysorb strips were coated with 100 µl of antigen preparation in each well and incubated at room temperature overnight. Each well was then washed four times with wash buffer (PBS with 0·05% sodium azide and 0·1% Tween-20). Plates coated with LPS-core were donated by Dr GR Barclay, Blood Transfusion Service, Edinburgh.[7] These were prepared using LPS purified by phenol/chloroform/petroleum extraction from four rough mutant bacteria, *Salmonella typhimurium* Rc878, *Escherichia coli* K12, *Klebsiella aerogenes* M10B, and *Pseudomonas aeruginosa* PAC605. Each was made up to a concentration of 0·2 mM in water and complexed with 0·4 mM polymixin B. Equal volumes of each antigen were mixed to give a final LPS-core concentration of 0·1 mM and 100 µl per well was dispensed into Nuclon maxisorb plates, incubated overnight at room temperature, and washed as above. PBS containing 5% bovine serum albumin (100 µl) was then added to each well, incubated overnight at room temperature, and washed as above. Plates were stored at −20°C.

URINE ANTIBODY ASSAYS

Urine samples (100 µl) were added in duplicate to microplate wells, incubated at 37°C for one hour, and washed four times. Alkaline phosphatase labelled anti-human IgG (ICN Immunologicals) was diluted 1 in 500 in ELISA diluent (PBS containing 0·05% Tween-20, 0·5% bovine serum albumin, 4% (w/v) polyethylene glycol 6000, and 0·05% sodium azide), and 100 µl added to each well, incubated for one hour at 37°C, and washed four times. P-nitrophenyl phosphate (100 µl of a 1 mg/ml solution: Sigma) in 0·05M sodium carbonate, 1mM magnesium chloride, pH 9·8, was added and the colour measured at 405 nm after 30 minutes at room temperature. Each assay included duplicate blank wells with no urine added, and duplicate reference wells containing standard human IgG (IgG from pooled blood donor sera, donated by Dr GR Barclay, diluted to 50 µg/ml in ELISA diluent and stored at −20°C). Wells without antigen but washed and post-coated as above were also used as negative controls.

ABSORPTION ASSAY

Samples (100 µl) of antibody positive urine were added in duplicate to wells coated with LPS-core antigen and incubated at 37°C for one hour. The samples were then transferred to Wells freshly coated with LPS-core antigen. Incubation and transfer were repated twice so that each sample had been incubated for a total of three hours in three separate wells. Antibody was then measured using MHCE coated plates. Antibody results were compared with those for unabsorbed urine and urine that had been absorbed in control wells (wells containing no LPS-core antigen but washed and post-coated with albumin in the same way as LPS-core coated wells).

STATISTICAL ANALYSES

Data were analysed using the Statistical Package for Social Sciences (SPSS-PC) (Information Analysis Systems).

Results

For all but one of the asymptomatic controls, antibody results (OD_{405}) were $<0·1$ with both MHCE and LPS-core antigens. One male asymptomatic control did have consistently high titres of urinary antibody to both antigens (OD_{405} $>1·0$). He did not have covert urinary tract infection, haematuria, proteinuria, hypertension, or any other evidence of renal or other disease.

Of the 72 specimens from symptomatic patients, 19 were culture positive ($>10^5$ cfu/ml, all with coliforms), and the remaining 53 were culture negative ($<10^4$ cfu/ml). Pus cells were seen in 17 of the 19 culture positive specimens, and in one of the 53 culture negative specimens.

IgG antibody was detected by both LPS-core ELISA and MHCE in the urine samples of most of the symptomatic patients (fig 1).

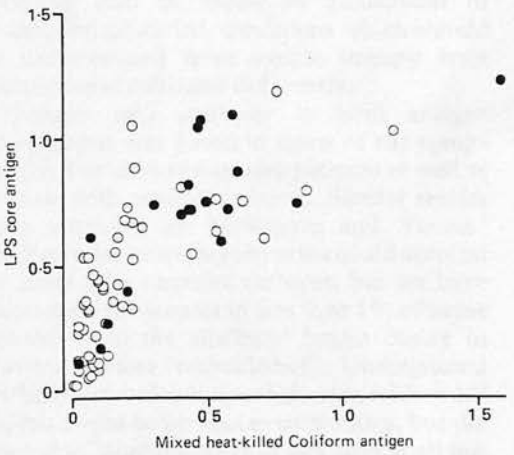

Figure 1 Correlation of IgG antibody (OD) with mixed, heat-killed coliform and LPS-core antigens in 72 urine samples from symptomatic patients ● = culture positive; ○ = culture negative.

Table 1 IgG antibody (OD) to individual and mixed LPS-core antigens in eight positive urine samples

Sample No	Mixed	Antibody titres (OD_{405}) detected by LPS-core from			
		Klebsiella aerogenes	*Pseudomonas aeruginosa*	*E coli*	*Salmonella typhimurium*
1	1·346	0·393	0·435	0·440	0·535
2	1·310	1·087	1·135	1·077	1·012
3	0·293	0·154	0·135	0·140	0·205
4	0·992	0·758	0·719	0·587	0·722
5	0·969	0·340	0·452	0·400	0·528
6	0·888	0·507	0·480	0·513	0·473
7	0·995	0·463	0·478	0·514	0·672
8	1·302	0·358	0·256	0·300	0·676

Antibody positive specimens from symptomatic patients had detectable titres of antibody against all of the individual bacteria which went to make up the MHCE antigen, as found by McKenzie and Young.[5] Similarly, the individual LPS-core antigens permitted the detection of antibody (table 1).

For the symptomatic patients there was a significant correlation between antibody to LPS-core antigen and antibody to MHCE antigen ($r = 0{\cdot}7633$, $p < 0{\cdot}001$). Within this group there was also a significant association between antibody titre and positive culture for both LPS-core antigen ($t = 3{\cdot}65$; $p < 0{\cdot}001$) and MHCE antigen ($t = 3{\cdot}55$; $p < 0{\cdot}001$). The predictive value of the antibody tests for positive culture was, however, low. For LPS-core antigen, an OD value of >0·6 OD predicted positive culture with a sensitivity of 15/19 (79%) and a specificity of 15/30 (50%). For the MHCE antigen an OD of >0·4 predicted positive culture with a sensitivity of 13/19 (68%) and a specificity of 13/21 (62%).

The correlation between the two assays suggested that they were measuring the same antibody. This was supported by the demonstration that the antibody detected by the MHCE antigen was reduced following absorption by LPS-core antigen in coated wells (fig 2).

Because LPS-core antigen is present only in Gram negative bacteria it might be predicted that this antibody would not be found in urinary tract infection caused by Gram positive organisms. Antibody to both antigens, however, was found in all six urine samples from symptomatic patients from which $>10^5$ Gram positive organisms were grown in pure culture (table 2).

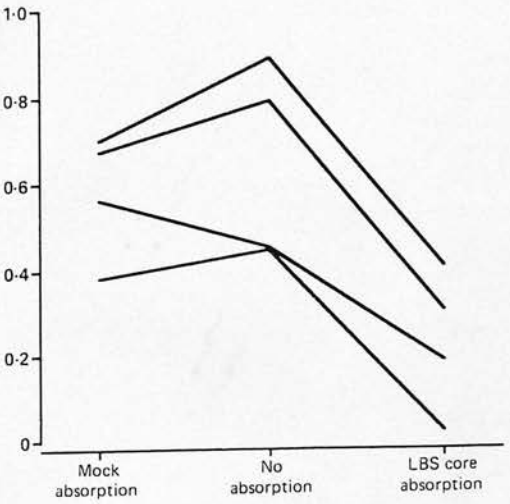

Figure 2 Reduction in IgG antibody (OD) to mixed heat-killed coliform antigen following absorption with LPS-core antigen or mock absorption on blank plates.

Discussion

The results presented here show that the presence of urinary IgG antibody detected in the MHCE assay is correlated with the presence of anti-LPS-core IgG. The assays could be measuring two quite separate antibodies which happen to occur together, or they could be measuring the same antibody. The demonstration that the coliform antibody can be reduced by absorption with LPS-core antigen suggests that antibody to LPS-core antigen makes up a sizeable proportion of the antibody detected by the MHCE antigen. This in part reflects the fact that other antigens in the MHCE assay will have been denatured by heat (in the case of proteins) or will be highly strain specific (in the case of 0 antigens). It may also indicate that antibody to LPS-core is an important part of the host immune response in urinary tract infection, as it is thought to be in septic shock.[8]

The simplest explanation for the presence of IgG core and coliform antibody in urine during urinary tract infection would be that inflammation and leakage of plasma proteins results in the presence in urine of unselected IgG antibodies from serum, as is thought to occur in pyelonephritis.[4] This would explain why the antibody is present early in the course of infection, when patients are presenting to their GP with symptoms. A specific immune response to infecting organisms would not be expected to result in an antibody response at this stage. It would also explain why the antibody is not a specific response to the infecting organisms, as is seen in the Gram positive infections. Such antibody would probably also be found in gonococcal or chlamydial urethritis, conditions which should be differentiated from simple urinary tract infection and managed differently.

Urinary IgG antibody to both antigen preparations was found in many of the symptomatic but culture negative patients as well as in those with positive cultures. Similar results were obtained by McKenzie and Young.[5] Antibacterial substances in urine could account for some false negative cultures, but we have found such substances in less than 1% of urine samples from the students' health centre in previous studies (unpublished). Undiagnosed urethritis, or urinary tract infection with $<10^4$ cfu/ml, might be present in some cases, but the absence of large numbers of pus cells in all but one of the culture negative specimens militates against this.[9] There is no satisfactory explanation for the presence of acute urinary symp-

Table 2 IgG antibody (OD) to mixed heat-killed coliform and LPS-core antigen in six urine samples with Gram positive infection

Sample No	Cultured Gram positive organism	Antibody titre (OD_{405}) detected by	
		MHCE antigen	LPS-core antigen
1	*Enterococcus*	0·256	0·571
2	*Enterococcus*	0·589	1·054
3	*Staphylococcus saprophyticus*	0·265	0·584
4	*Staphylococcus saprophyticus*	0·100	0·227
5	*Staphylococcus saprophyticus*	0·688	1·235
8	*Group G Streptococcus*	0·528	1·091

toms in many patients, however,[9 10] and it is therefore impossible to relate these findings to established underlying mechanisms. The presence of antibody does suggest that the symptoms have a physiological basis.

The purpose of measuring urinary antibody to a mixture of common urinary pathogens[5] was that it would permit rapid detection of urinary tract infection and would be less sensitive to contamination or overgrowth than is conventional bacteriology. In our hands IgG coliform or core antibody did not predict positive culture in fresh, carefully taken specimens to any useful extent. Such antibody tests are therefore not likely to be useful in examining contaminated specimens or specimens which have been delayed, or where the patients have been on antibiotics. Other workers have found that measuring lipopolysaccharide antibodies in urine is not a useful way of screening urine samples before culture.[6]

It is clear that urinary antibodies can be measured in association with symptoms suggestive of urinary tract infection. It may be possible to define particular classes or specificities of urinary antibody which are of predictive value in the diagnosis of urinary tract infection. There is, however, much to be learned about the relation between urinary tract infection and urinary antibodies before such a test can be devised.

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Frequencies of Lipopolysaccharide Core Types among Clinical Isolates of *Escherichia coli* Defined with Monoclonal Antibodies

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Mouse monoclonal antibodies (MAbs) specific for the lipopolysaccharide (LPS) core types R1, R2, and R3 of *Escherichia coli* and a cross-reactive MAb that binds to the LPS core of almost all *E. coli* were used in ELISA to determine the frequency of cores resembling R1, R2, and R3 in strains of *E. coli* isolated from clinical samples (blood and urine specimens) and from the feces of asymptomatic individuals. Of the 180 wild-type isolates, 123 were assigned to R1 core type, 14 to R2, and 18 to R3. Twenty-five wild-type *E. coli* isolates could not be assigned to a particular core type and may have either an R4 or K12 core or a previously unrecognized core type. R1 core type was associated with O types 1, 4, 6, 8, and 18 and with K1 or K5 capsules. R3 was associated with O15. O75 isolates could be of either R1 or R2 core type.

The lipopolysaccharide (LPS) core region of *Escherichia coli* has a conserved overall structure, with an inner 2-keto-3-deoxy-octonate–heptose region and an outer hexose region. Most of the variation that does occur is in the outer part of the core, furthest from lipid A. In wild-type smooth *E. coli*, the core region can occur in at least four different forms, known as R1, R2, R3, and R4 [1–3]. The naturally rough strain K12 has a different core region [4].

The O antigen of smooth LPS may mask some of the epitopes and phage attachment sites in the core region. The chemical, serologic, and phage typing methods that have been used to define the different core types of *E. coli* are therefore not directly applicable to wild-type smooth strains. The frequency with which these different core types occur in wild-type *E. coli* is therefore not known.

Little is known about the association between core types and O serotypes of *E. coli*, except that the original R1 and R2 rough mutants were both derived from O8 strains [1] and that the original R3 mutant was derived from an O111 strain [2]. There is no published evidence on the relationship of LPS core types to capsular types or to virulence. Information about the distribution of O and K antigens has proved to be useful, for example in identifying and understanding enteropathogenic strains [5] and in suggesting novel therapeutic approaches to bacteremia [6]. Information about core types might be equally interesting.

We therefore set out to produce a panel of mouse monoclonal antibodies (MAbs) that would react with specific LPS core types of *E. coli* and other gram-negative bacilli with related core structures. We wished to use these MAbs as a means of determining the LPS core types of a collection of wild-type *E. coli*.

Materials and Methods

Bacteria and bacteriophages. *E. coli* R1 (F470), R2 (F576), R3 (F653), R4 (2513) and K12 (2131) and rough-specific phages FO, Br10, C21, 6SR, and T4 were obtained from G. Schmidt (Forschunginstitut, Borstel, Germany). *E. coli* O1, O2, O4, O6, O12, O15, O18, and O75 were obtained from A. S. Cross (Walter Reed Army Institute, Washington, DC). Phages specific for *E. coli* capsular types K1 (ϕK1GS) and K5 (ϕK5DG) were obtained from A. P. Roberts (Charing Cross and Westminster Medical School, London). Blood culture and urine isolates were obtained from the Clinical Bacteriology Laboratory, Department of Medical Microbiology, Edinburgh University, from routine clinical specimens. The blood culture isolates were all of the gram-negative organisms isolated in a 1-year period. Twenty-one fecal isolates of *E. coli* were obtained, each from a separate healthy volunteer. Rough mutants of *Salmonella minnesota* (R60), *Salmonella typhimurium* (1542), *Klebsiella pneumoniae* (M10B), and *Pseudomonas aeruginosa* (PAC608) and wild-type smooth *Shigella sonnei* (colicin types 4/2 and 2/1) and *Shigella flexneri* (serotypes 1a and 3) were freeze-dried stock cultures held in our laboratory.

MAbs. MAbs were produced by standard methods [7]. The details of immunization schedules and selection procedures are to be published elsewhere. All were reactive in ELISA with polymyxin-complexed rough LPS [8]. All MAb preparations were supernatants of hybridoma cell cultures grown in RPMI 1640 supplemented with 5% fetal calf serum. Antibody concentration was in the range of 10–50 μg/mL.

ELISA. Overnight nutrient broth cultures of bacteria were washed and resuspended in saline to give an OD of 0.5 at 525 nm and heated for 30 min at 100°C. Cells were then diluted

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1/20 in coating buffer (sodium carbonate-bicarbonate, pH 9.6, with 0.05% sodium azide) and 100 μL/well was added to Nunc (Roskilde, Denmark) Polysorb strips. After overnight incubation at room temperature, plates were washed three times with wash buffer (0.05% Tween 20 and 0.05% sodium azide in PBS, pH 7.4) and postcoated with 5% (wt/vol) bovine serum albumin (BSA) in coating buffer overnight at room temperature. Plates were then washed and stored at −20°C.

MAbs were diluted 1/250 in dilution buffer (4% [wt/vol] polyethylene glycol, 0.05% sodium azide, and 0.05% tween 20 in PBS, pH 7.4). Diluted MAb (100 μL) was added to wells and incubated for 90 min at 37°C. Plates were washed three times, and urease-conjugated anti-mouse immunoglobulin (Zymed, Cambridge, UK; diluted 1/500 in dilution buffer with 0.5% BSA, 100 μL/well) was added and incubated for a further 90 min. Plates were then washed three times in wash buffer and three times in distilled water; urease substrate solution (Seralab, Crawley Down, UK; 100 μL/well) was added and plates were incubated for 90 min at room temperature and read at 590 nm. An OD of >0.8 was considered positive.

PAGE and immunoblotting. LPS was prepared by proteinase K (protease type XI; Sigma, Poole, UK) digestion of lysed bacteria [9] and separated on 14% polyacrylamide gels [10] with SDS omitted from stacking and separating buffers. Gels were then silver stained [9] or blotted to nitrocellulose membrane (0.2-μm pore size) [11]. Blots were blocked with 3% (wt/vol) gelatin in TRIS-buffered saline, pH 7.5 (TBS), incubated for 3 h in a 1/10 dilution of MAb in 1% gelatin in TBS followed by a 1-h incubation with horseradish peroxidase anti-mouse IgG conjugate (ICN, High Wycombe, UK; diluted 1/1000), and developed with horseradish peroxidase color reagent (Bio-Rad, Hemel Hempstead, UK).

Serum sensitivity of bacteria. Serum from one donor was stored at −70°C. This serum had IgG antibodies to LPS core at a level equal to the 65th centile of the normal population [8]. Overnight broth cultures were washed and resuspended in complement fixation test (CFT) buffer (Oxoid, Basingstoke, UK) at room temperature to an OD of 0.5 at 525 nm. This suspension was then diluted 1/5000 in CFT buffer, and 10 μL was added to 70 μL of CFT buffer and 20 μL of freshly thawed serum or heated (56°C, 30 min) serum. After 1 h at 37°C, duplicate 20-μL samples were spread on the surface of nutrient agar plates, and colonies were counted after overnight incubation. If the number of colony-forming units in the fresh serum was <10% of the number in the heated serum, the organism was considered to be serum sensitive.

O typing. *E. coli* isolates from blood and urine cultures were screened for common O antigens, by ELISA with absorbed O typing sera (Difco, Detroit) to O1, O2, O4, O6, O7, O12, O15, O18, O22, and O75 (diluted 1/1000) and by tube agglutination at a final dilution of 1/80 with unabsorbed sera to O8 and O9 (Statens Seruminstitut, Copenhagen). O types were confirmed by conventional tube agglutination [12].

Phage typing. Nutrient agar plates were surface inoculated by flooding with overnight broth culture and allowed to dry. Phage suspension (10 μL) was dropped onto the agar and incubated overnight. A clear zone of lysis was scored as sensitive.

Table 1. Reactivity of selected monoclonal antibodies (MAbs) in ELISA.

	Reactivity with *E. coli* strain							
MAb	R1	R2	R3	R4	K12	O12	O15	O18
SZ 43 3.4.8	+	−	−	−	−	−	−	+
H4 361.23	−	+	−	−	−	+	−	−
W4 434.07	−	−	+	−	−	−	+	−
SZ 27 150.3	+	+	+	+	+	+	+	+

NOTE. +, strong reactivity (OD >0.8); −, no or weak reactivity (OD <0.15).

Statistics. All *P* values are two-tailed results calculated by Fisher's exact test.

Results

Selection of core-specific MAbs. A preselected panel of LPS-reactive MAbs was tested by ELISA against heat-killed *E. coli* rough strains R1, R2, R3, R4, and K12; *E. coli* smooth strains O1, O2, O4, O6, O12, O15, O18, and O75; and the rough mutants of *K. pneumoniae*, *P. aeruginosa*, *S. minnesota*, and *S. typhimurium*. MAbs that reacted with a restricted range of R types and also with some smooth strains of *E. coli* were thought likely to be useful in core typing wild-type smooth strains. Four such distinct patterns of reactivity were observed with more than one antibody. R1-specific MAbs (e.g., SZ 43 3.4.8) reacted with R1 and with *E. coli* O1, O2, O4, O6, O18, and O75; R2-specific MAbs (e.g., H4 361.23) reacted with R2 and O12; R3-specific MAbs (e.g., W4 434.07) reacted with R3 and O15; cross-reactive MAb SZ 27 150.3 reacted with all the *E. coli* rough and smooth strains and with the *Salmonella* rough mutants but not with the *Klebsiella* or *Pseudomonas* rough mutants (table 1).

Other patterns were observed, including a group of MAbs that reacted with R2 and K12, with R2 and the *Salmonella* rough mutants, or with all of these strains. However, no MAb reacted with K12 or R4 but not with other rough mutants. None of the MAbs used in this study reacted with the *Klebsiella* or *Pseudomonas* rough mutants tested.

The specificity of MAbs for LPS core was confirmed, for the MAbs given as examples above, by immunoblotting against LPS (proteinase K extracts) from *E. coli* R1, R2, R3, R4, K12, O12, O15, and O18. The O12, O15, and O18 strains were chosen as they seemed, from the ELISA data, to represent smooth strains with R2, R3, and R1 core types, respectively. As seen in figure 1, the immunoblots showed reactivity of each MAb with a band corresponding to the LPS core of each of the organisms with which it reacted in ELISA.

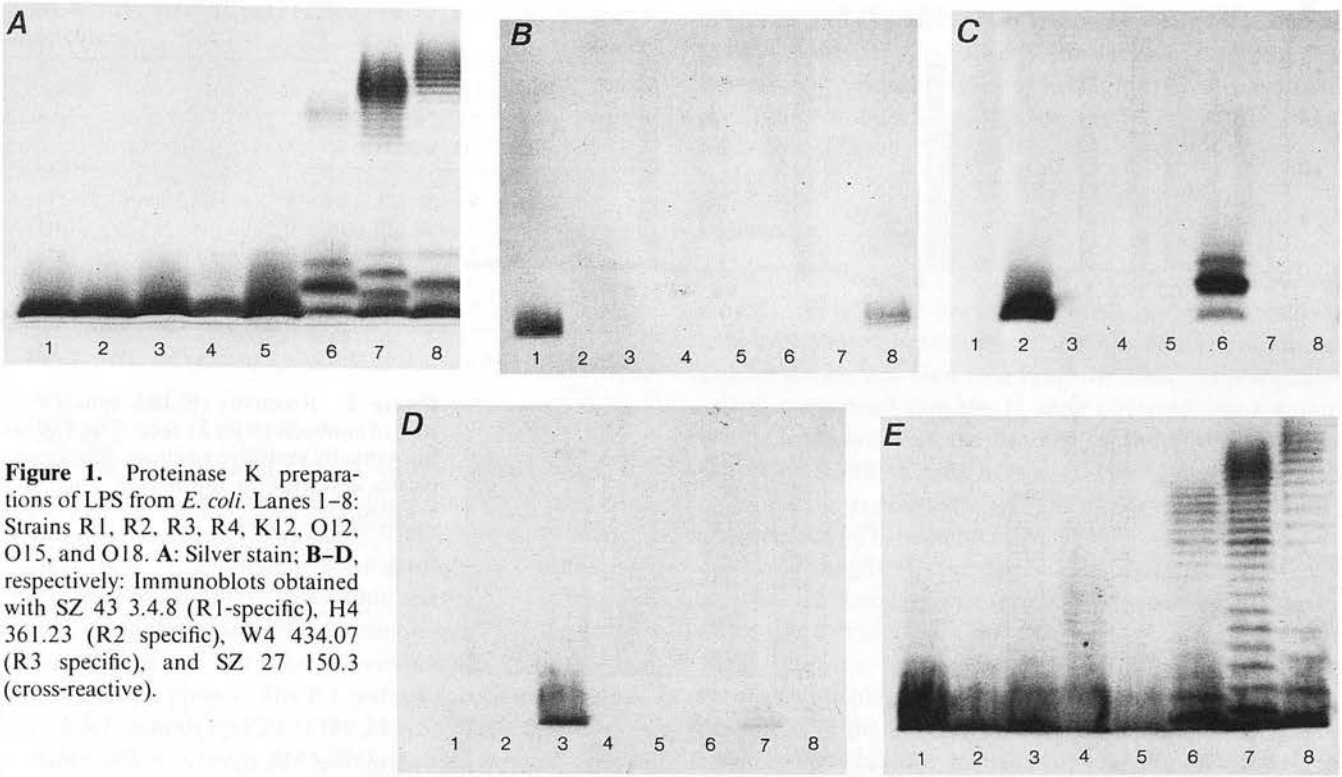

Figure 1. Proteinase K preparations of LPS from *E. coli*. Lanes 1–8: Strains R1, R2, R3, R4, K12, O12, O15, and O18. **A**: Silver stain; **B–D**, respectively: Immunoblots obtained with SZ 43 3.4.8 (R1-specific), H4 361.23 (R2 specific), W4 434.07 (R3 specific), and SZ 27 150.3 (cross-reactive).

With the rough mutants, a band was seen that corresponded to the single band seen in the silver stain. With the smooth strains, the R1 and R3 MAbs again reacted with a single band corresponding to the fastest-moving band seen in the silver stain. This band consists of LPS core that has not been substituted with O antigen [13]. The R1 antibody reacted in the same way in blots with the O1, O2, O4, O6, and O75 strains (not shown). The R2-specific MAb H4 361.23 and the cross-reactive MAb SZ 27 150.3 reacted with the unsubstituted core band and also with the "ladder" pattern of bands corresponding to O-substituted LPS core. MAb SZ 27 150.3 reacted weakly in the blot with bands in the R1 and R4 LPS preparations that were not visible in the silver stain. We believe these bands represent production, due to a leaky mutation, of small quantities of O-substituted LPS that are not detected by the silver stain but are detected by immunoblot.

Binding of anti–LPS core MAbs to gram-negative bacilli other than E. coli. Four *Shigella* strains were tested in ELISA, and all reacted with the cross-reactive MAb. In addition, both *S. sonnei* strains reacted with the R1-specific MAb, while the *S. flexneri* strains reacted with the R3-specific MAb. This is in agreement with previous studies of LPS core in these species [14–16]. Blood culture isolates of other gram-negative genera (*Acinetobacter*, 7; *Citrobacter*, 5; *Enterobacter*, 18; *Klebsiella*, 14; *Proteus*, 9; *Providencia*, 1; *Pseudomonas*, 8; *Salmonella*, 2; and *Serratia*, 5) were also tested in ELISA with the core type–specific and cross-reactive MAbs. The only positive reaction observed was between the two *Salmonella* isolates and the cross-reactive MAb. The cross-reactive MAb SZ 27 150.3 was tested in an immunoblot against LPS (proteinase K extracts) from single blood culture isolates of *K. pneumoniae*, *Proteus mirabilis*, *P. aeruginosa*, *Enterobacter cloacae*, and *Citrobacter freundii*. No reaction was observed with any of these isolates, confirming the lack of reactivity seen in ELISA.

Frequency of MAb-defined core types in wild-type isolates of E. coli. *E. coli* isolates from blood cultures (n = 79), urine cultures (n = 80), and fecal specimens from asymptomatic volunteers (n = 21) were tested in ELISA with core type–specific and cross-reactive MAbs. The cross-reactive MAb reacted with all of the isolates tested except for two of the urine isolates. When the strains reacting with the cross-reactive MAb were tested with the R1-, R2-, and R3-specific MAbs, each MAb reacted with a mutually exclusive group of isolates, which were classified as R1, R2, and R3, respectively. Figure 2 illustrates these results for the fecal isolates. Of the two urine isolates that did not react with the cross-reactive antibody, one reacted with the R1 antibody. These and the isolates that did not react with any of the core-specific antibodies are referred to as RNC (not classified). The numbers of wild-type isolates in the four categories are given in table 2. R1 was the most common classification overall (123/181, 68%) and was more common among isolates from

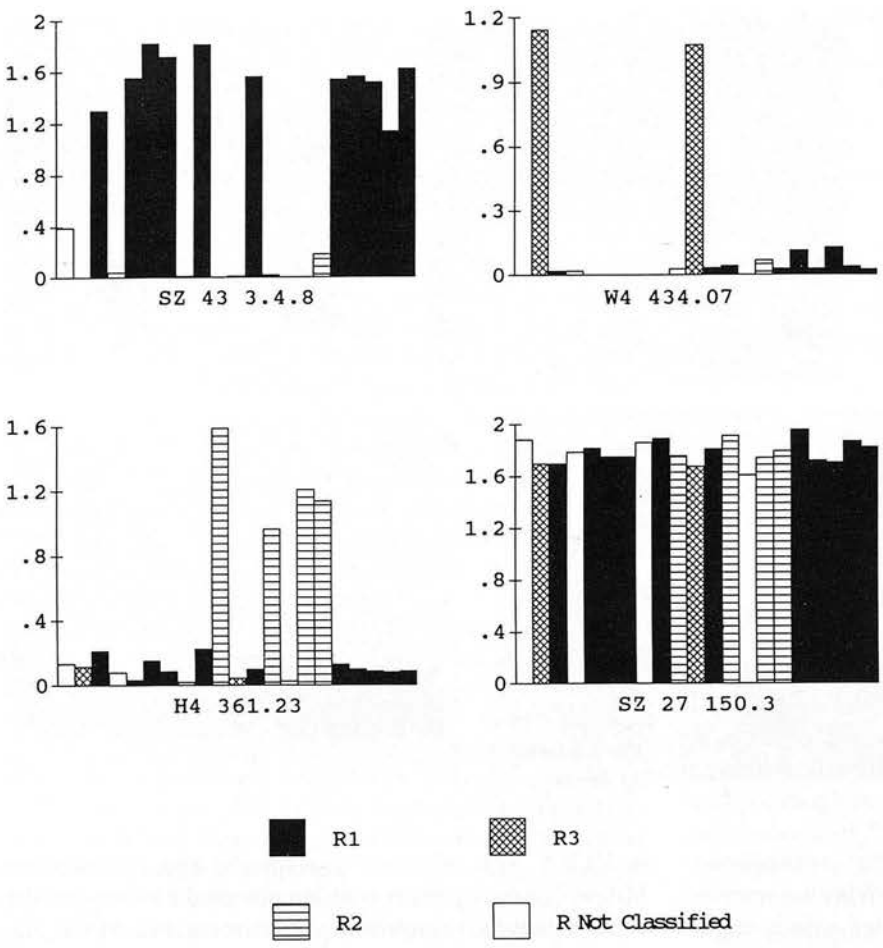

Figure 2. Reactivity (ELISA optical density) of antibodies with 21 fecal *E. coli* showing mutually exclusive reactions. Shading indicates core type to which each strain was assigned.

urine (62/80, 78%) than among those from blood culture (48/79, 61%, $P = .017$) or feces (11/21, 52%, $P = .030$).

Those MAbs that had reacted with R2, K12, and *Salmonella* rough mutants reacted with all of the R2 group of wild-type isolates but also reacted with variable numbers of the RNC isolates. This showed that the RNC group was heterogeneous but did not permit clear subdivision of this group.

Relationship between MAb-defined core type and sensitivity to rough-specific and K-specific phages (table 3). The collection of wild-type *E. coli* was tested for sensitivity to the rough-specific phages that have been used to distinguish between the different R types of *E. coli* [3], since it was thought that the pattern of reactivity might support the MAb-defined R typing scheme. Fifty of the 180 isolates tested were sensitive to one or more of the rough-specific phages. They were found among each of the R types defined with MAbs. They included isolates that were O typeable and produced a ladder

Table 2. Frequency of core types in wild-type isolates of *E. coli* determined by binding of core-type–specific monoclonal antibodies (MAbs) in ELISA.

Source	No. (%) of strains assigned to MAb-defined core type				
	R1	R2	R3	RNC	Total
Blood culture	48 (61)	6 (7)	12 (15)	13 (16)	79
Urine	64 (81)	4 (5)	4 (5)	8 (10)	80
Feces	11 (52)	4 (19)	2 (10)	4 (19)	21
Total	123 (68)	14 (8)	18 (10)	25 (14)	180

NOTE. NC, not classified.

Table 3. Sensitivity of 180 *E. coli* of different core types to rough-specific, K1-specific, and K5-specific phages.

Core type (*n*)	No. of strains sensitive to phage							
	F0	Br10	C21	6SR	T4	Any R+	K1	K5
R1 (123)	0	8	17	5	28	40	19	20
R2 (14)	0	2	0	1	3	3	0	1
R3 (18)	0	0	0	0	2	2	0	1
RNC (25)	0	3	2	2	3	5	3	2
Total (180)	0	13	19	8	36	50	22	24

NOTE. Any R+, any rough-specific phage; NC, not classified.

Table 4. O type of *E. coli* of different core types from blood and urine cultures.

Core type (*n*)	No. of each core type associated with O type										
	O1	O2	O4	O5	O6	O7	O8	O15	O18	O75	NT
R1 (112)	11	0	6	1	26	3	3	0	10	6	46
R2 (10)	0	0	0	0	0	0	0	0	0	3	7
R3 (16)	0	0	0	0	0	0	0	3	0	0	13
RNC (21)	1	2	0	0	0	2	0	0	0	0	16
Total (159)	12	2	6	1	26	5	3	3	10	9	82

NOTE. NT, not typed by available O-specific sera; NC, not classified.

pattern on silver stain of LPS, as was found by Cross et al. [6]. C21 sensitivity, a marker for the R1 rough mutant, was found in 19 isolates (17 R1 and 2 RNC). FO sensitivity is a marker for R2 rough mutants, but no isolate was FO sensitive. There was no other discernible relationship between R type and sensitivity to particular rough-specific phages.

Forty-six of the 180 isolates were sensitive to one of the two K-specific phages. The R1 isolates accounted for 19 of the 22 K1 isolates and 20 of the 24 K5 isolates found.

Relationship between MAb-defined core type and O type among the urine and blood culture isolates. The urine and blood culture isolates of *E. coli* were tested for 12 O types found commonly among *E. coli* from blood culture [6, 12] or urinary tract infection [17] (table 4). The overall proportion of isolates that belonged to one of these O types was much higher among those of R1 core type (66/112, 58%) than among the other core types (11/47, 23%, $P = .0005$). Some constant associations between O type and core type were apparent: all 26 O6 isolates were R1, and all 3 O15 isolates were R3. In contrast, O75 isolates were found among both the R1 and the R2 core types. The proportion of the R1 isolates that belonged to one of the common O types was greater among the blood culture isolates (32/48, 67%) than among the urine culture isolates (34/64, 53%). This difference is not statistically significant ($P = .177$), but the number of R1 isolates not belonging to the common O types could account for the greater proportion of R1 isolates among the isolates from urine.

Relationship between R type, serum resistance, and LPS chemotype among blood culture isolates. Serum resistance, which is a property of most invasive strains of *E. coli* [6], was determined for all the blood culture isolates; 21 (26%) of 79 were serum sensitive. There were serum-sensitive isolates of all the core types, but serum sensitivity was slightly less common among the R1 isolates (9/48, 19%) than among the other R types (10/31, 32%). This difference may have been due to the association of R1 core with K1 and K5 capsule, since all of the 11 K1 isolates and all but 1 of the 10 K5 isolates from blood cultures were serum resistant.

Seventy of the 79 blood culture isolates produced a typical ladder pattern of LPS in silver stains of proteinase K digests (data not shown). The other 9 showed only one or a few fast-moving bands, one corresponding to the unsubstituted core. Three isolates were R1, 3 were R2, 2 were R3, and 1 was RNC.

Discussion

We have described a group of MAbs that are selective for the LPS core of *E. coli*. Some of the MAbs also bind to *Salmonella* and *Shigella* strains that share *E. coli* core structures. The MAbs do not bind to a range of other gram-negative bacilli, confirming that the binding observed was specific. We have shown that the binding of these antibodies in ELISA to bacterial cells heated at 100°C corresponds to their binding to unsubstituted (and in some cases O-substituted) LPS core in immunoblots.

Unsubstituted LPS core is present in smooth bacterial cells but may not be readily accessible to MAbs in standard laboratory cultures [18, 19]. Core epitopes are, however, much more accessible on bacterial cells grown in serum or in a magnesium-depleted medium, which is thought to mimic the conditions in the infected host [20]. Core epitopes also become more accessible in bacterial preparations heated at 100°C [21, 22]. The expression and accessibility of core epitopes and the possible existence of cross-reactive epitopes have important implications for immunotherapy of gram-negative sepsis [23].

We have examined the binding of selected MAbs to heated bacterial cells in ELISA. We could recognize three mutually exclusive groups among *E. coli* isolates and labeled them R1, R2, and R3 after the corresponding rough mutants. A fourth group, which could not be assigned to a particular core type, was referred to as RNC and is probably of heterogeneous core type. We included in the RNC group the strain that reacted with the R1-specific antibody but not with the cross-reactive MAb. If this strain had been of R1 core type, a reaction with the cross-reactive MAb would have been expected.

We anticipate that each of the groups R1, R2, and R3 defined with MAbs probably has the core structure of the named rough strain, though this has not been confirmed by chemical analysis. The presence of C21 sensitivity (a marker for R1 rough mutants) among the wild-type isolates of MAb-defined R1 rather than R2 or R3 core type supports our view that the MAb-defined core types are the same as the reference rough strains. Two RNC isolates were also C21 sensitive, but the specificity of these phages for particular core types is not based on an independent knowledge of their receptor sites, and it is not known how they might react with as-yet-unidentified core types of *E. coli*. Phage typing is of little value in determining the core type of wild-type isolates since most are resistant to all of the phages used.

No isolates of R4 or K12 core type were positively identi-

fied. This may be explained by the lack of suitable specific MAbs. We did have MAbs reactive with K12, but these also reacted with R2 and did not bind to a clearly defined group of wild-type isolates other than those that reacted with the R2-specific MAbs. The RNC group may include isolates with R4 or K12 core but probably also contains one or more as-yet-unidentified core types.

Comparison of the structural formulas [15] with the specificities of our MAbs could suggest binding of the MAbs to particular sugar residues. R1 and R3 each have a unique terminal branching hexosyl unit (β1-3–linked glucose and α1-3–linked *N*-acetylglucosamine, respectively) that may be the epitope for the R1 and R3 antibodies. R2 and *Salmonella* core share a terminal α1-2–linked *N*-acetylglucosamine, while R2 and K12 have in common the next three sugar residues. These may therefore be the epitopes for the MAbs that bound to these combinations of strains. A unique binding site for the R2-specific antibody is not obvious from the structural formulas. From the calculated three-dimensional structure of the different core types [24], however, it appears that the branch-terminating α1-6 galactose present in R2, *Salmonella* core, and K12 may be in a different orientation in R2 and may therefore be the R2-specific epitope. It is of note that while most of the core-specific MAbs reacted in immunoblot only with unsubstituted core, the R2-specific MAb also reacted with O-substituted core, giving a ladder pattern in immunoblots. This implies that the R2-specific epitope is the only one not altered or masked by the attachment of the O polysaccharide. Further study of the binding site of these antibodies may therefore be useful in elucidating the three-dimensional structure of LPS core and in attempts to define the attachment site of the O polysaccharide.

The binding of the cross-reactive MAb SZ 27 150.3 to almost all of the *E. coli* isolates and to the *Salmonella* and *Shigella* strains, together with the pattern of binding of the core-specific MAbs, supports the view that these organisms share closely related LPS cores. The lack of binding of any of the antibodies to other genera suggests that these core structures are not distributed more widely. The structure of LPS core has not been determined for all the other genera, but it has been shown that the LPS cores of *Citrobacter* [25, 26] *Proteus* [27], and *Pseudomonas* [28] species are different from that of *E. coli*.

A MAb designated T6, which recognized the R2 core of *E. coli* and the core of *Salmonella* species, did not react with 25 clinical isolates of *E. coli* in Hong Kong [29], suggesting that the R2 core type was rare. We have found, however, that R2 accounted for 8% of our collection of *E. coli* isolates. This suggests that attempts to use MAb T6 in a capture ELISA [22] to detect *Salmonella* organisms in clinical samples may yield a significant number of false-positives because of the presence of *E. coli* of R2 core type.

The tendency for certain apparently distinct properties of *E. coli* to occur together [5] seems to apply to the relationship between core types and O and K types. It may be that there are chemical or structural factors limiting the ability of *E. coli* to express certain combinations of O and core types (e.g., O15 and R1) or it may be that some such combinations have a selective advantage in the host. The finding that there was greater proportion of R1 core type among the urine isolates and in particular that there was a greater proportion of R1 isolates not belonging to one of the common O types may indicate some relationship between R1 core type and unusual O types and pathogenicity in the urinary tract. Further studies are needed before firm conclusions can be drawn on this point.

Core typing with MAbs may be a useful addition to current typing methods for *E. coli*. The number of possible types is small, and the core type does not usually discriminate among strains of a given O and K type, but core typing may be useful in recognizing relatedness between groups of *E. coli*. We hope to use these antibodies to investigate the LPS core type of a wider range of *Shigella* and *E. coli* isolates from a variety of sources.

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REVIEWS IN MEDICAL MICROBIOLOGY

The role of bacteria in sepsis syndrome

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Summary. The name 'sepsis syndrome' has been suggested for a precisely defined severe generalized illness in which underlying infection is suspected. Endotoxin is thought to play a key part in its pathogenesis. The syndrome has a high mortality despite antibiotics and intensive care. A range of new therapies aimed at endotoxin, and the cytokines which it induces, is being developed and tested. Among patients with sepsis syndrome the group with bacteraemia do not have a worse outcome, probably because this is the group which obtains most benefit from antibiotics. Anti-endotoxin antibody treatments tested so far seem to be of benefit mainly or exclusively to those patients with Gram-negative bacteraemia, implying that the role of endotoxin is greatest in these patients. Protein toxins and peptidoglycan fragments could produce the features of sepsis syndrome in some patients, but this possibility has not been explored because there are no suitable assays. In a substantial number of patients neither endotoxin nor bacterial infection can be shown to be involved in the pathogenesis of sepsis syndrome.

Introduction

Clinical definitions

The term 'sepsis syndrome' has been used[1–3] to describe a condition of fever or hypothermia associated with tachypnoea, tachycardia and evidence of inadequate tissue perfusion in a patient with clinical evidence of infection. 'Septic shock' is used to refer to a more severely ill group of patients who, in addition to the features noted above, have signs of shock. These definitions could apply to a very wide range of patients, but are usually applied to patients in intensive care units in developed countries. Most such patients are suffering primarily from trauma, or from intra-abdominal infection which has not been eradicated surgically. Classical infectious diseases which might produce similar clinical effects (and which might share similar underlying mechanisms of pathogenesis) are excluded, either explicitly (in the case of tuberculosis[4]), or implicitly because of the clinical setting in which this term is applied.

The definition of sepsis syndrome is by no means standardized. Terms such as septicaemia,[5,6] systemic sepsis[7] and sepsis[8,9] have been used to describe the condition in broadly similar groups of patients. Others have used 'sepsis' applied only to those patients with pus[10] or microbiological evidence of infection.[8,11] The argument is far from settled.[8,12] The fundamental problem with this example of clinical taxonomy, as in some areas of microbiological taxonomy, is that definitions have been based on empirical observations and not supported by a complete understanding of the underlying processes. Sepsis syndrome certainly includes many patients with classical Gram-negative sepsis[13] but also includes patients with a similar clinical presentation in whom bacterial infection is not the apparent cause.

Despite its difficulties and necessarily arbitrary nature, classification of patients based on features which can be determined quickly and easily is necessary in sepsis syndrome if rapid therapeutic action is to be taken. Until recently, treatment of these patients has consisted of surgery, physiological support and antibiotics. Antibiotic treatment is often given to patients in whom infection is not subsequently confirmed microbiologically, but this is accepted because of the expected benefit from antibiotics in patients with Gram-negative bacteraemia,[14,15]

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Table Organisms isolated from blood cultures from 223 patients with sepsis syndrome[7]

Organism	Number of patients
Escherichia coli	15
Klebsiella pneumoniae	10
Enterobacter spp.	4
Serratia spp.	4
Proteus spp.	9
Pseudomonas spp.	3
Other Gram-negative spp.	6
Staphylococcus aureus	11
Staphylococcus epidermidis	4
Streptococcus pneumoniae	11
Other streptococci	6
Other Gram-positive spp.	4
Candida spp.	3
No organism isolated	133

coupled with the time required to make a definite microbiological diagnosis and the lack of evidence that antibiotics could be harmful. When new treatments aimed at controlling the deleterious aspects of the host response are tested[7,9,11,16,17] it is necessary to review their effect on all patients who would receive such treatments. Many more such treatments aimed at different aspects of the host response are being developed,[3] and will also need to be tested in this way. The concepts of sepsis syndrome and septic shock are therefore set to remain with us, despite their limitations.

Aims of this review

My purpose in this article is to review the role of bacteria in sepsis syndrome and septic shock. I have used the term 'sepsis syndrome' to refer to the generalized illness outlined above. Because no standard definition is in use, it is inevitable that the meaning of the term will vary according to the study under discussion. I have also used the term 'septic shock' to refer to the more severely ill group of patients who have developed shock, but again the definition is not standardized. Following the suggestion of Bone,[1] I have not used the term 'septicaemia' because of the confusion over its meaning. As well as series of patients with sepsis syndrome, I have drawn on series of patients who have been selected because they have bacteraemia. The nature of the clinical illness in these patients is even more variable, and is not always clearly defined. These differences create real problems and uncertainties in analysis of the data, but until a large body of data using standard case-definitions is produced these problems are unavoidable.

Interpretation of bacteriological results

The organisms generally thought to be involved in sepsis syndrome may all be found living as commensals in or on intensive care patients (Table).[7] These patients are often colonized by organisms such as *Pseudomonas*, which are uncommon in healthy individuals, and are often colonized in sites such as the upper respiratory tract by organisms normally restricted to the colon.[18] These organisms may go on to cause infection, but it is not possible to conclude that they are acting as pathogens simply because they have been isolated from a sick patient. It is necessary to consider whether the organism has invaded a normally sterile site, or whether bacterial products (notably endotoxin) may be having deleterious effects without invasion.

Bacterial invasion

Bacteraemia is the most easily interpreted bacteriological finding in sepsis syndrome patients. Blood can be sampled easily and aseptically in every patient, and any organisms cultured from the blood have clearly invaded the host, though the problem of contamination of specimens must always be remembered. The only other similar specimen is cerebrospinal fluid (CSF), which is unlikely to be invaded in most patients with sepsis syndrome.

In some patients there may be organisms from other sites, such as abscess cavities or the peritoneum, which can be regarded as clear evidence of invasion. Cases where such isolates are made are relatively few, however. These cases are not easily comparable with one another and are therefore difficult to analyse in large series of patients with sepsis syndrome. In the case of peritonitis there is also the potential for confusion between faecal contamination from the initial perforation and the organisms which go on to cause infection.[19] Infection at other sites, notably the respiratory tract, undoubtedly occurs in intensive care patients and is associated with high mortality, but diagnosis is very imprecise, and has not been related to the aetiology of sepsis syndrome.[20]

Attention has therefore focussed on bacteraemia as the most readily understood way of documenting infection in these patients. This does not mean that bacteria must be in the blood to cause sepsis syndrome; it is simply more difficult to be sure of the role of organisms from other sites.

Endotoxaemia

As well as detecting whole bacteria, it may be possible to infer microbial involvement in an illness by detecting bacterial toxins, notably endotoxin, in the blood. Endotoxin is certainly capable of producing the features of sepsis syndrome, and a great deal of research has focussed on this area.[3,21,22] Endotoxin assays based on Limulus amoebocyte lysate (LAL) were originally developed as a way of detecting rapidly the presence of Gram-negative bacteria, and positive results in the absence of bacteria were

initially regarded as false-positives.[23] More recently it has been noted that endotoxaemia is an independent prognostic indicator, and indeed a better indicator for the development of shock[5] or death[24] than bacteraemia. Endotoxin may be absorbed from the gut[25] rather than being derived from organisms in the blood or other sites of infection. Endotoxaemia is therefore not necessarily evidence of bacterial infection.

Antibody response as evidence of infection

Another general approach to the diagnosis of infection is to show a rising titre of antibody to the infecting organism. This approach is complicated in sepsis syndrome. The organisms involved form part of the normal commensal flora, and therefore many patients have high levels of antibody prior to the septic episode. High levels of pre-existing antibody do not confer complete immunity, but are associated with a good outcome in Gram-negative bacteraemia.[26] Many patients die before a detectable antibody response would be expected. Looking for a rising antibody titre has been found to be of no value in diagnosis of *Escherichia coli* bacteraemia.[27]

Bacterial invasion in sepsis syndrome

There have been four major prospective studies of patients with clinically defined sepsis syndrome[9,11,17,28] and one of septic shock.[24] Studies of sub-groups of three of the sepsis syndrome series have been published separately, duplicating some of the data.[4,7,29] Two other studies[16,30] were prospective in design but appear to have included Gram-negative bacteraemia as one of the possible entry criteria and cannot therefore be used to determine the frequency of bacteraemia in sepsis syndrome.

Bacteraemia

In prospective series of sepsis syndrome patients, bacteraemia was found in 40–47%[17,28] and Gram-negative bacteraemia was found in 21–37%.[9,11,17,28] In the prospective study of septic shock, 39% had bacteraemia, and 19% had Gram-negative bacteraemia.[24] Full detail of the blood culturing protocol is not given in any of these studies, and there may be false negatives in some series if inadequate volumes of blood were cultured before antibiotics were given.[31] Bacteraemia (Gram-positive or Gram-negative) was associated with the development of septic shock in one series of febrile patients[5] and in one series of sepsis syndrome patients,[4] but not in the other series. Bacteraemia was not associated with a worse outcome in any of these series.

Since bacteraemia is not associated with a worse outcome in sepsis syndrome, one is forced to question its significance. Studies of patients with bacteraemia, particularly Gram-negative bacteraemia, have shown high mortality rates,[15,32] but the bacteraemia in these patients might just be an epiphenomenon, with the real harm coming from gut-derived endotoxin or some other stimulus to the cytokine release. What evidence is there that bacterial invasion is harmful?

Evidence that bacterial invasion is harmful

There is evidence derived from clinical studies of the effect of antibiotics to support the idea that bacterial invasion is harmful. Since the demonstration that early antibiotic treatment led to greatly reduced mortality in *Pseudomonas* bacteraemia,[14] antibiotics have been used empirically for patients in whom bacteraemia is suspected. It would be considered unethical now to withhold antibiotics as part of a double-blind study to show their benefit, and therefore such definitive studies have not been done. More oblique approaches have been used, however, including comparisons of different antibiotic regimes and retrospective studies of the outcomes of individual patients.

Comparison of antibiotic regimens

Large studies in febrile neutropenic patients have compared different empirical antibiotic regimens[13,33] and have shown differences between outcome for the different regimens. These studies have also shown that in-vitro sensitivity tests on blood-culture isolates are good predictors of the effectiveness of treatment regimens. This implies that the differences in outcome observed are due to the antibiotic action of the drugs, and therefore that the living bacteria detected in the blood were important. However, in these patients, unlike sepsis syndrome patients, bacteraemia itself is a predictor of a worse outcome.[33] This difference is probably related to the much lower clinical threshold for admission of these patients to trials. These results therefore confirm that bacteraemia can be a cause of illness, but they are not necessarily applicable to sepsis syndrome patients in whom other more powerful insults may be responsible for the illness observed.

Retrospective series of bacteraemic patients

Retrospective studies have looked at the difference in outcome for patients treated with appropriate antibiotics (antibiotics to which the isolated organism was sensitive) and those treated with inappropriate antibiotics (to which the organism was not sensitive). Significantly lower mortality rates in patients receiving appropriate antibiotics were found in three large series of bacteraemic patients[15,32,34] and in several smaller series of patients bacteraemic with individual organisms such as *Enterobacter*,[35] *Klebsiella*,[36], *E. coli*[37] and enterococci.[38] The clinical state of these patients was not defined, however, so that, as in the comparative studies above, they would not all have

had 'sepsis syndrome'. The relationship between appropriate antibiotic and outcome has been considered in one small study of sepsis syndrome patients,[39] which confirmed that inappropriate antibiotics were associated with a significantly worse outcome. Delay in introduction of the appropriate antibiotic was also associated with a significant increase in hospital stay and with the development of acute organ failure, though not with increased mortality. This study considered appropriateness in relation to 'the cultured pathogen', which presumably means isolates from abscesses and other sites as well as blood cultures, but nevertheless supports the view that antibiotics can be effective in sepsis syndrome patients, and therefore that invading organisms, such as blood-culture isolates, are important.

Do antibiotics completely neutralize the deleterious effect of bacterial invasion?

The apparent effectiveness of antibiotic treatment offers an explanation for the failure to observe a worse outcome in sepsis syndrome patients who have bacteraemia. Among sepsis syndrome patients, 90.5–98%[9,11,16] of those with Gram-negative bacteraemia, or 86–91% of all patients with significant positive cultures,[7,39] received appropriate therapy. The lack of difference between bacteraemic and non-bacteraemic patients with sepsis syndrome could therefore be because the deleterious effect of bacteraemia is largely neutralized by effective antibiotics.

There does, however, seem to be room for further improvement in the outcome of sepsis syndrome patients, despite adequate antibiotics. Ziegler et al[9] showed that the human monoclonal IgM antibody HA-1A reduced the mortality rate from 49% to 30% in patients with sepsis syndrome who had Gram-negative bacteraemia, although the mortality rate rose from 40% to 45%[40] in those patients who did not have Gram-negative bacteraemia. The benefit was greatest in patients who had Gram-negative bacteraemia and shock. No benefit was reported in the group of patients who had Gram-negative infection (defined by culture from the site of infection) but no bacteraemia. There is some doubt about how exactly HA-1A might work,[41,42] but the demonstration that it is only active in patients with Gram-negative bacteraemia suggests that these patients are different in an important respect from other sepsis syndrome patients. This is true even after the beneficial effect of antibiotics has been taken into account.

Slightly different results have been obtained in studies with two other antibody treatments[11,16] in patients with sepsis syndrome, both of which showed benefit in patients with Gram-negative infection. Most of these patients had bacteraemia, but a proportion ranging from 10%[16] to 46%[11] had infection defined only by isolates from the site of infection. The patients with non-bacteraemic Gram-negative infection appeared to benefit from the antibodies used in these studies, although the results do not reach statistical significance for the non-bacteraemic subgroup because of the small numbers involved. In the first of these studies,[16] using polyclonal human anti-J5 serum, patients with Gram-negative infection confirmed by isolates from specimens other than blood were only included in the analysis if the negative blood culture could be 'explained' by the fact that appropriate antibiotics were given before blood cultures were taken. Other patients who did not have Gram-negative bacteraemia were excluded from the analysis, and presumably did not benefit. In the second study, using the murine monoclonal IgM anti-endotoxin antibody E5,[11] patients with Gram-negative infection, whether bacteraemic or not, appeared to benefit from antibody treatment if they were not in septic shock. The relationship between negative blood-cultures and prior antibiotic treatment is not discussed in this study. No benefit was found in the patients without Gram-negative infection in the E5 study.

The role of shock in these studies is interesting. Both of the studies by the Ziegler group[9,16] showed greatest benefit from antibody treatment in patients with shock, consistent with the idea that the antibody is neutralizing endotoxin and therefore favouring reversal of shock. In contrast, the E5 study[11] found no benefit in shock patients. These differences may be explained by differences in the action of the antibodies, or by differences in the exact definitions of shock, or by other unrecognised differences in the types of patient studied.

The conclusions of these studies also differ in respect to the non-bacteraemic patients, as discussed above, but they share some conclusions that are relevant to this review. They all show that there is potential for improved outcome in sepsis syndrome patients with Gram-negative infection in addition to what is achieved by appropriate antibiotics and other conventional therapies. They also support the idea that there are important differences between sepsis syndrome patients with bacterial infection and those without. Other studies that show no benefit from antibody treatment[30] do not detract from these conclusions.

Endotoxaemia in sepsis syndrome

It is remarkable, in view of the central role which endotoxin is thought to play in sepsis syndrome,[3] how few of the large studies of sepsis syndrome have reported the results of endotoxin assays. This is a reflection of the major practical difficulties in actually performing the assays,[43] particularly in the multi-centre type of studies which have been required to collect large numbers of sepsis syndrome patients. Danner et al[24] measured endotoxaemia in septic shock and showed that it was associated with a poorer prognosis. They also found that 57 of 100 patients with septic shock did not have endotoxaemia, despite

performing an average of 9.6 endotoxin assays per patient. Either the assays were missing transient episodes of endotoxaemia, or some other mechanism is involved in these patients. This implies that endotoxaemia cannot be confidently regarded as the sine qua non of septic shock.

Antibiotics and endotoxin release

The finding that antibiotics are of benefit in bacteraemia runs contrary to the suggestion that antibiotic treatment might lead to release of endotoxin and therefore to worsening of clinical progress.[44] The evidence that significant endotoxin release happens in vivo, however, is weak.

Experiments in rabbits have shown an increase in the ratio of endotoxin to bacteria in the blood following therapy with moxalactam[45] or gentamicin.[44] In these experiments bacteraemia was induced by injecting 20ml of overnight broth culture of *E. coli*, with mucin, into the peritoneum. The reported rise in endotoxin:bacteria ratio in the treated animals can be explained largely by the fact that antibiotic caused a reduction in the number of viable bacteria in the blood, rather than any marked difference in the amount of endotoxin in the blood. The endotoxin level did continue to rise after antibiotic treatment, but was no greater in the treated animals than in the controls. Endotoxin may well have continued to be absorbed from the very large inoculum (10^9–10^{10} c.f.u.) in the peritoneum.

In humans, it has been clearly shown in meningococcal infection that endotoxin levels in the blood decline consistently and rapidly following the introduction of antibiotic therapy.[46] Rising levels of endotoxaemia have been observed during the first few hours of antibiotic therapy in some patients with septic shock[24] or Gram-negative bacteraemia,[47] but these rising levels have not been consistently associated with poor outcome.

How does bacterial invasion lead to disease?

The triggering of cytokine release by endotoxin derived from the invading organisms is the best-known mechanism by which bacterial invasion may lead to clinical sepsis syndrome,[3] but is only relevant to Gram-negative infections.

Endotoxin in the blood is not necessarily derived from invading bacteria, but may in many cases be absorbed or 'translocated' from the gut.[25] This is presumably the mechanism which leads to endotoxaemia in Gram-positive infection,[5,47] but begs the question of how bacterial invasion leads to endotoxin translocation. Mechanisms other than the effects of endotoxin must be involved in sepsis caused by Gram-positive infection, and may well be involved in Gram-negative infections.

A group of protein toxins produced by *Staphylococcus aureus, Streptococcus pyogenes* and some other organisms are now recognized as 'super antigens' which can stimulate T-cells via the V_β element of the T-cell receptor.[48] This stimulation triggers release of tumour necrosis factor (TNF) and interleukin-1 (IL-1), and can produce systemic illness in animal models. Toxic shock syndrome is a well-recognized manifestation of this effect in humans and would, in many cases satisfy the clinical criteria for sepsis syndrome.

Other bacterial components are able to induce the features of sepsis syndrome, though with relatively low specific activity. Cell walls of Gram-positive bacteria are pyrogenic,[49] and low molecular weight peptidoglycan is probably the active component. Muramyl dipeptide (MDP) is a synthetic molecule which appears to be the minimum structure required to mimic the effect of low-molecular-weight peptidoglycan, and its activity has been studied in some detail.[50] MDP induced the production of endogenous pyrogen (IL-1) from rabbit white blood cells or human mononuclear cells in vitro. The action of MDP did not appear to be due to endotoxin contamination of the material, since LAL assays were negative, and cross-tolerance experiments suggest that the mechanism of action of MDP is different from that of endotoxin.

Neither 'super antigen' protein toxins nor peptidoglycan fragments can be readily detected in clinical samples. The importance of these factors in sepsis syndrome has therefore not been explored, but this does not mean that they are unimportant.

Conclusions

There is no doubt that live bacteria and bacterial products, particularly endotoxin, can be, and sometimes are, responsible for the features of sepsis syndrome. What is much less clear is the relative importance of live bacteria, endotoxin and other bacterial products in these patients. To answer the fundamental questions, and to guide the wide range of new therapies being developed, we need methods as sensitive as LAL to recognize other microbial products and methods to recognize bacteraemia more quickly. Equally important is the identification of non-microbial causes of sepsis syndrome, together with rapid methods to recognize them.

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