

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

2

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information, including suggestions for reducing this burden. Send comments to Washington, DC 20503.

AD-A277 811



Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information, including suggestions for reducing this burden. Send comments to Washington, DC 20503.

BERS

1. AGENCY USE ONLY (Leave blank) 2. REPORT DATE

4. TITLE AND SUBTITLE

Therapeutic intervening in sepsis with antibody to endotoxin: is there a future?

6. AUTHOR(S)

A. CROSS & S. OPAL

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Walter Reed Army Institute of Research
Washington, DC. 20307-5100

8. PERFORMING ORGANIZATION
REPORT NUMBER

9. SPONSORING, MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Research & Development Command
Ft. Detrick, MD. 21702-5012

10. SPONSORING, MONITORING
AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

DTIC
S E D
ELECTE
APR 05 1994

12a. DISTRIBUTION AVAILABILITY STATEMENT

APPROVED FOR PUBLIC RELEASE:
DISTRIBUTION UNLIMITED

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

Within the last 24 months a number of studies that tested the efficacy of immunologic reagents in the treatment of sepsis were concluded. Among these reports were 4 studies¹⁻⁴ completed on 2 anti-endotoxin monoclonal antibodies (MAb, HA-1A and E-5). These clinical trials did not generate data sufficient to support product licensure. Given the attention and expectations surrounding the anti-endotoxin MABs, the disappointing results raised the question whether the use of anti-endotoxin antibodies in the treatment of sepsis was still a viable concept.⁵ The question was rendered even more relevant given the decade-old controversy surrounding the efficacy of polyclonal antibodies to endotoxin, particularly antibody to the J5 (Rc chemotype) mutant of *Escherichia coli* O111:B4, a conceptual progenitor of the HA-1A and E-5 MABs.⁶ In this review we shall examine whether anti-endotoxin antibodies may yet offer any therapeutic potential in the treatment of sepsis. It will be our contention that antibodies to core glycolipid will be useful adjuncts to therapy, particularly if used as part of combination immunotherapy.

DTIC QUALITY INSPECTION

14. SUBJECT TERMS

sepsis

15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION
OF REPORT18. SECURITY CLASSIFICATION
OF THIS PAGE19. SECURITY CLASSIFICATION
OF ABSTRACT

20. LIMITATION OF ABSTRACT

94-10163

1518



JOURNAL OF ENDOTOXIN RESEARCH

Minireview

Therapeutic intervention in sepsis with antibody to endotoxin: is there a future?

A. S. Cross, S. Opal

Department of Bacterial Disease, Walter Reed Army Institute of Research, Washington DC, USA, and Division of Infectious Diseases, Memorial Hospital of Rhode Island, Department of Medicine, Brown University School of Medicine, Providence, RI, USA

Availability Codes	
Dist	Avail and/or Special
A-1	20

SUMMARY. Within the last 24 months a number of studies that tested the efficacy of immunologic reagents in the treatment of sepsis were concluded. Among these reports were 4 studies¹⁻⁴ completed on 2 anti-endotoxin monoclonal antibodies (MAb, HA-1A and E-5). These clinical trials did not generate data sufficient to support product licensure. Given the attention and expectations surrounding the anti-endotoxin MAbs, the disappointing results raised the question whether the use of anti-endotoxin antibodies in the treatment of sepsis was still a viable concept.⁵ The question was rendered even more relevant given the decade-old controversy surrounding the efficacy of polyclonal antibodies to endotoxin, particularly antibody to the J5 (Rc chemotype) mutant of *Escherichia coli* O111:B4, a conceptual progenitor of the HA-1A and E-5 MAbs.⁶ In this review we shall examine whether anti-endotoxin antibodies may yet offer any therapeutic potential in the treatment of sepsis. It will be our contention that antibodies to core glycolipid will be useful adjuncts to therapy, particularly if used as part of combination immunotherapy.

HISTORICAL OVERVIEW

Endotoxin, or pyrogen, had been described since the mid-19th century, and was the subject of intensive investigation since the 1920s. Many of the observations of the clinical responses to endotoxin, including those after infusions in man,⁷ were an outgrowth of studies on typhoid immunization. Extra-intestinal infections with less virulent enteric bacilli were relatively uncommon. Although bacteremia with Gram-negative bacilli in man had been well described since the 1920s,⁸ the clinical syndrome of Gram-negative bacterial sepsis was not described until the early 1950s.^{9,10} Nevertheless, extra-intestinally invasive infection with opportunistic Gram-negative bacilli was not recognized as a significant clinical problem for nearly a decade later. At that

time, landmark reviews by Finland¹¹ and Rogers¹² documented the ascendancy of these infections, particularly among hospitalized patients.

The interval between initial clinical descriptions of Gram-negative bacillary sepsis and the recognition of the increased significance of Gram-negative bacillary infections, however, was marked by considerable investigation of the pathogenic properties of these opportunistic bacteria, especially *E. coli*. The post-World War II outbreaks of *E. coli* diarrhea led to studies that addressed the virulence determinants of this organism.^{13,14} The discovery of properdin by Pillemer energized a number of investigators to re-examine the role of serum in bacteriolysis. The result of these efforts was to identify the lipopolysaccharide phenotype and the capsular polysaccharide as important to the virulence of Gram-negative bacilli in experimental systems. The clinical relevance of these experimental findings was established when it was observed that Gram-negative bacteria cultured from the blood of patients were overwhelmingly serum-resistant.¹⁵ A milestone was achieved with the elucidation of the structural

A. S. Cross MD, Department of Bacterial Diseases, Walter Reed Army Institute of Research, Washington DC 20307-5100, USA, S. Opal MD, Division of Infectious Diseases, Memorial Hospital of Rhode Island, Department of Medicine, Brown University School of Medicine, Providence, RI, USA.

Correspondence to Dr Alan S. Cross.

DTIC QUALITY INSPECTED 94 4 4 071

features of Gram-negative bacterial lipopolysaccharide through both biochemical and bacterial genetic studies.¹⁶⁻¹⁸ The essential features of lipopolysaccharide (LPS, or endotoxin), namely the common, toxic lipid A moiety, an inner core sugar region and an antigenically distinct O polysaccharide repeat unit were identified, with little subsequent change to this day.

Although many antimicrobial agents had activity against Gram-negative bacilli, the pace of discovery of antibacterial agents with activity against these pathogens increased with the development of new aminoglycoside antimicrobials and extended spectrum penicillins. Nevertheless, despite these new antibiotics, there was still an unacceptable mortality from Gram-negative bacterial sepsis. With the improved care and longer survival of immunocompromised patients, the incidence of this syndrome increased. Consequently, immunotherapeutic and immunoprophylactic measures to neutralize the toxic properties of endotoxin were sought. Initially, because of the apparent heterogeneity of species and multiple serotypes of pathogenic Gram-negative bacilli, such an approach was considered not feasible, since antibodies directed against the outermost O polysaccharide provided homologous, but not heterologous protection in animal models; however, as the structure of endotoxin was elucidated, it was apparent that among *Enterobacteriaceae* and *Pseudomonas* there was a highly conserved core glycolipid region. Consequently, many investigators considered the generation of an antibody response to the common core glycolipid region of LPS to be a reasonable experimental approach.

DEVELOPMENT OF POLYCLONAL ANTI-CORE GLYCOLIPID ANTIBODY

Experimental studies

Localized *E. coli* infection in animals was found to cause sustained endotoxemia in the absence of bacteremia, and induced a protective antibody response to the endotoxin independent of O antibody formation;¹⁹ therefore, Tate et al stimulated such anti-endotoxin antibody by immunization with a boiled whole cell vaccine from a rough LPS mutant of *E. coli* O113.²⁰ Similar rough LPS vaccines that unmasked a common core region were prepared from mutants of *E. coli* O111 which were unable to utilize UDP-galactose for the formation of distal O polysaccharide. The antiserum obtained from immunization with this vaccine was able to prevent LPS-mediated toxicity, as manifest by (1) a lowering of mortality rate in experimental animals following LPS infusion, and (2) significant reduction in both local (dermal) and generalized (DIC) Schwartzman reactions initiated by LPS (reviewed in²¹). This antiserum was also able to protect against lethal bacteremia caused by *Klebsiella*, *Pseudomonas* and *E. coli* in a neutropenic rabbit model of sepsis.^{22,23} The absence of anti-O antibody in the protective antisera and the inability of

antisera prepared from the parental O111 strain to inhibit these responses were taken as further evidence of the importance of the core epitopes.

In concomitant studies Chedid et al found that immunization of animals with a rough mutant of *Salmonella typhimurium* protected against lethal challenge with *Klebsiella pneumoniae*. He proposed that antibodies to rough determinants of LPS can protect against infection with Gram-negative bacilli having a smooth LPS phenotype.²⁴

In an extensive series of studies, McCabe and colleagues found that both active and passive immunization with an Re mutant of *Salmonella minnesota* whose cell wall contains only KDO and lipid A also afforded heterologous protection in experimental infection, but such protection was not observed following immunization with lipid A.²⁵⁻²⁷ These studies by Chedid, Braude, McCabe and colleagues all lent support to the concept that antibodies to endotoxin might provide either therapeutic and/or prophylactic benefit to patients at risk of Gram-negative bacterial sepsis.

Clinical studies

Based on these studies, a study was performed in 136 humans to assess the efficacy of J5 antiserum in the treatment of Gram-negative bacterial sepsis.²⁸ Of 83 patients who had culture-documented Gram-negative infections and received optimal conventional therapy (102 with bacteremia and 16 critically ill patients with local infection in whom antibiotics had already been started), mortality was reduced nearly 50% (26% with non-immune serum and 14% with J5 antiserum, $P = 0.16$). Among the 18 patients whose hypotension required pressors for at least 6 h, 2/7 (29%) of controls and 9/11 (82%) recipients of J5 antiserum recovered from shock ($P = 0.024$). It was not possible to demonstrate that the efficacy of J5 antiserum in this study was correlated to J5 antibody levels in the patients. A subsequent study evaluated the ability of J5 antiserum to prevent Gram-negative shock and death in surgical patients at high risk of Gram-negative infection. Prophylaxis with J5 antiserum significantly decreased the incidence of shock and more significantly, death from shock (relative risk in controls was 2.3 and 4.2 overall, and higher in those with abdominal surgery); however, as in the previous study, it had no effect on the incidence of infection. Again, the ameliorative effect of J5 antiserum was not correlated with levels of J5-specific antibody in the patients.²⁹

In a later study, the prophylactic administration of a single dose of J5 antiserum to patients with neutropenia did not reduce the number of febrile days, the number of Gram-negative bacteremic episodes or death from these infections.³⁰ This result should not have been unanticipated since results of the initial clinical study²⁸ and an experimental study³¹ each indicated that J5 antiserum had little effect on the acquisition of infection.

RECENT STUDIES WITH POLYCLONAL ANTI-CORE GLYCOLIPID ANTIBODY

Since the J5 antisera used in the early clinical trials could not be mass-produced in a safe, standardized manner, it was unlikely that it would ever have been licensed for general use. Consequently, these human studies might be regarded more as clinical studies designed to test a concept rather than as phase III studies in support of a potential biologic product. With the development of immunoglobulins for intravenous administration (IVIG), studies were performed to evaluate various IVIGs, both standard commercial preparations as well as preparations enriched in antibody to Gram-negative bacilli, in the prophylaxis or treatment of Gram-negative sepsis.

Standard IVIG

The therapeutic administration of standard IVIG at 400 mg/kg at entry and at 2 and 5 days did not result in a significant increase in survival in one 24 patient study.³² In a study of 55 patients, Schedel and colleagues administered as therapy for septic shock a polyclonal, non-hyperimmune immunoglobulin preparation that contained IgG, IgA and IgM isotypes (Pentaglobin, Biotest, Dreieich, Germany). The statistically significant decrease in septic mortality (1/27 vs 9/28, $P < 0.01$) was correlated with a decrease in circulating endotoxin activity and maintenance of levels of IgG antibody to lipid A following IVIG therapy, although the study was not designed to show whether specific anti-endotoxin antibodies accounted for the reduced mortality.³³

IVIG screened for anti-core glycolipid antibody

An earlier study had shown that plasma screened for natural antibody to a panel of Gram-negative bacterial antigens resulted in a 7-fold decrease in mortality when administered as therapy for septic shock in an obstetrical/gynecology ward.³⁴ Based on a similar screening of blood donor plasma against the core LPS of *S. minnesota* R595, a core LPS antibody-enriched IVIG was compared to standard IVIG at doses of 400 mg/kg for its ability to prevent serious Gram-negative bacteremic complications in patients admitted to a surgical intensive care unit.³⁵ Interestingly, among the 329 evaluable patients, those receiving the standard (non-immune IVIG) had fewer cases of Gram-negative bacterial pneumonia than those receiving either the core hyperimmune globulin or albumin placebo. There was no difference in the incidence of systemic infection, shock or mortality. Since the core antibody-enriched preparation should differ from the standard IVIG only in the amount of anti-core glycolipid antibody, it is puzzling that patients receiving the anti-glycolipid antibody fared worse than those receiving standard IVIG. A polyvalent IgG (Nordimmun) has been developed from plasma screened for antibodies to a panel of LPS antigens;

however, to our knowledge, it has not been tested in a double-blind, placebo-controlled prospective study.³⁶

Vaccine-induced anti-core glycolipid IVIG

A J5 IVIG was prepared from the plasma of donors immunized with a *E. coli* J5 vaccine according to previously successful protocols; however, in a therapeutic study of 100 patients, treatment with a single intravenous dose of 200 mg/kg J5-IVIG was as ineffective as standard IVIG in reducing mortality or in reversing shock.³⁷

A study of children with severe infectious purpura found that treatment with post-J5 immunization plasma (i.e. not IVIG) had no effect on the clinical course or mortality.³⁸

Anti-endotoxin monoclonal antibodies

With the advent of monoclonal antibody (MAb) technology it was thought that a series of unambiguous experiments should have been able to confirm or refute the validity of the core glycolipid antibody hypothesis, and to eliminate lingering doubts about the role of antibody and the precise molecular mechanism of protection of polyclonal J5 antisera.³⁹⁻⁴¹ Unfortunately, many of the same immunologic, biochemical and physiologic factors that preclude a consensus opinion on polyclonal J5 antisera also have applied to the anti-endotoxin MABs^{42,43} (see below).

While many MABs to core glycolipid structures have been described in the literature, two antibodies, E5 (Xoma, Berkeley, CA, USA)⁴⁴ and HA-1A (Centocor, Malvern, PA, USA)⁴⁵ have been investigated in both preclinical and clinical studies of sepsis.^{2,46-49} The E5 MAB is a typical murine MAB isolated from murine ascitic fluid, while the HA-1A MAB is a human MAB produced in a human-mouse heteromyeloma fusion system. In the latter instance, a patient who was to undergo staging laparotomy for Hodgkin's disease was immunized preoperatively with an *E. coli* J5 vaccine. Isolated splenocytes were then harvested to produce the hybridoma fusion partners, resulting in a MAB which consisted of primarily human components.

In vitro binding activity

Both E5 and HA-1A bind to rough and smooth LPS, including heterologous LPS serotypes with complete O-specific side chains.⁵⁰ While both antibodies bind to the lipid A component of the core glycolipid structure with comparable binding avidities, competitive binding experiments and anti-idiotypic MAB blocking experiments suggest that each MAB binds to a separate epitope on the lipid A molecule. Non-specific, low affinity, binding to nucleic acids has also been reported.^{51,52} Using fluid phase radio-immunoassay, Warren et al⁵³ have shown the both MABs bind slightly to smooth LPS molecules of different serotypes only when the antibodies are present in high concentrations.

The ability of these MABs to bind to heterologous smooth LPS serotypes, however, does not ensure that they would bind to viable bacteria or bacterial cell wall remnants where the lipid A target is buried within the outer membrane and covered with O-specific polysaccharide, acidic capsular (K) polysaccharide and other outer membrane components. In this instance, the concomitant administration of bacteriocidal antibiotics have unmasked the core structure and allowed binding of the MABs.^{44,45,54}

In vitro functional activity

Binding a specific epitope does not necessarily indicate that neutralization or interference with the toxic properties of lipid A will occur. Neither MAB has convincingly demonstrated the capacity to inhibit the recognition of LPS by the Limulus lysate assay.⁵³ Moreover, attenuation of the proinflammatory cytokine response to LPS by the anti-endotoxin MABs has not been observed in either in vitro or in vivo systems.^{5,53} These observations cast doubt on the therapeutic relevance of these MABs in the treatment of septic shock.^{5,43,51,53}

Activity in animal models

MAB E5 improves the hemodynamics and physiologic parameters following endotoxin challenge in a sheep model.⁴⁶ This MAB was also able to provide modest protection from lethality, particularly when accompanied by antimicrobial agents, in bacteremic models in mice and rats.^{44,47} Survival benefits from the use of HA-1A were reported in neutropenic rabbit and mouse

peritonitis models.^{45,48} The HA-1A MAB also prevented the dermal Schwartzman reaction in rabbits.⁴⁵ In contrast, Baumgartner et al⁵ were unable to find a reduction in serum TNF levels, prevention of the dermal Schwartzman reaction or protection against LPS-induced lethality in galactosamine-treated mice with the use of HA-1A. Further, large doses of HA-1A (10 mg/kg, or approximately 8–10 times the dose used in the clinical trial) enhanced lethality in a Gram-negative bacteremia model in dogs.⁵⁵ If, however, the mechanism of HA-1A MAB action is its ability to promote the binding and clearance of endotoxin via CR1 receptors on human blood cells,⁵⁶ then no animal model, including sub-human primates, would be useful in the preclinical evaluation of this MAB. A recent clinical report suggests that HA-1A may facilitate endotoxin removal and diminish systemic TNF release in endotoxemic patients with sepsis.⁵⁷

Clinical trials

The initial phase III clinical trials with both MABs were reported in 1991 and have been extensively commented upon.^{42,58–60} Both MABs were studied in placebo-controlled, multicenter trials and enrolled patients using similar entry and exclusionary criteria (Table 1). While neither MAB provided a survival benefit to the entire study population when analyzed on an intent-to-treat analysis, nevertheless, both MABs did show a statistically significant survival benefit in certain subgroups. Unfortunately, the subgroups in which clinical efficacy was demonstrated differed between the two trials. This disparity in outcome analysis is difficult to reconcile as

Table 1. Comparison of results with initial phase III trials with E5 and HA-1A

	E5 (n = 468)			HA-1A (n = 543)		
	E5 (2 mg/kg x 2)	Placebo (5% dextrose)	P value	HA-1A (100 mg x 1)	Placebo (albumin)	P value
Total	NR (40%)*	NR (41%)	NS	262 (39%)	281 (43%)	NS
Age (mean)	60.1	64.3	< 0.05	58.0	62.3	NS
APACHE II (mean)	16.9	17.3†	NS	23.6	25.7	NS
% male	66%	66%	NS	59%	58%	NS
% in shock	55%	59%	NS	51%	51%	NS
% ARDS	20%	23%	NS	9%	13%	NS
% ARF	23%	22%	NS	35%	46%	NS
% DIC	29%	25%	NS	18%	21%	NS
Patients with GNB (n)	94	77	NS	105	95	NS
% mortality in GNB	NR	NR	–	30%	49%	0.014
% mortality GNB: shock	NR	NR	–	33%	57%	0.017
% mortality GNB: no shock	NR	NR	–	27%	40%	0.28
% mortality in GNI	38%	41%	NS	NR	NR	–
% mortality GNI: no shock	30%	43%	0.01	NR	NR	–
% mortality GNI: shock	45%	40%	NS	NR	NR	–

* 28-day all cause mortality rate (HA-1A); 30-day all-cause mortality rate (E5).

† APACHE II scores available from only 185 patients in the E5 trial.

ARDS: adult respiratory distress syndrome.

ARF: acute renal failure.

DIC: disseminated intravascular coagulation.

GNB: Gram-negative bacteremia.

GNI: Gram-negative infection.

NR: not reported.

NS: not significant.

both anti-endotoxin MABs were expected to function in a similar manner. HA-1A was observed to be beneficial in patients with Gram-negative bacteremia, particularly those who were in shock at study entry,⁴⁹ as in the first JS study.²⁸ The E5 MAB, on the other hand, appeared to be effective only in those patients who had Gram-negative sepsis in the absence of shock, whether or not the patients were bacteremic.² Further, resolution of sepsis-related multi-organ dysfunction (disseminated intravascular coagulation, adult respiratory distress syndrome, acute renal failure, and hepatobiliary dysfunction) was more commonly observed in those bacteremic patients who received HA-1A (62% HA-1A vs 42% placebo [$P = 0.024$]); or in patients who had Gram-negative sepsis and were not in shock who received E5 (54% E5 vs 30% placebo [$P = 0.05$]). Both MABs were well tolerated.

The results of these two trials generated considerable controversy and commentary as to their statistical validity, clinical applicability and economic feasibility. In this latter regard, an inability to rapidly identify those patients likely to respond to anti-endotoxin antibody treatment would necessitate administering the treatment to up to two-thirds of the septic population who met study entry criteria, yet who would not derive any benefit (patients without Gram-negative bacteremia in the HA-1A and patients without Gram-negative sepsis in the absence of shock in the E5). By some analyses, some patients who met study entry criteria but who did not fit into favorable subgroups might have done worse than the placebo group. Of the 331 patients in the HA-1A study, those who did not have Gram-negative bacteremia had a 45% mortality if they received HA-1A compared to a 40% mortality in the placebo group.^{49,61} Among the 179 patients in the E5 trial who had Gram-negative sepsis and were in shock, the mortality was 45% in the E5 and 40% in the placebo group.²

Because of these concerns, follow-up studies were performed with both MABs. In a trial designed to focus on those patients who appeared to respond to E5 treatment in the first study, 830 patients were enrolled over a 2-year period. Documented Gram-negative sepsis was present in 63% of patients and major organ failure was present in 30% of patients at study entry.⁴ This second trial failed to confirm a survival advantage with E5 therapy in this targeted group of patients: the 30-day all-cause mortality rate in patients with Gram-negative sepsis and organ dysfunction ($n = 139$) was 41% (E5) and 47% (placebo) ($P = \text{NS}$); however, resolution of organ dysfunction was significantly more likely to occur with E5 treatment, as was true in the first study.⁶² A third multicenter clinical trial with E5 is currently underway which will focus upon patients with Gram-negative sepsis and organ dysfunction and/or shock.

A second study with HA-1A (Centocor HA-1A Efficacy in Sepsis Study [CHESS Trial]) was designed to determine the efficacy of this MAB to reduce the 14-day all-cause mortality in patients with Gram-negative bacteremia. This study was terminated prematurely on safety considerations when the mortality rate in patients

without Gram-negative bacteremia was 41% with HA-1A compared to a 14-day mortality of 38% in placebo-treated patients. While this difference was not statistically significant ($P = 0.142$), the adverse trend resulted in discontinuation of the study after 2471 patients were enrolled. There was no statistically significant improvement in HA-1A treated patients with Gram-negative bacteremia.³ Based on these results, HA-1A was withdrawn from the European market. HA-1A continues to be studied in pediatric patients with meningococemia. This double-blind, placebo-controlled trial has enrolled 192 patients through December 1993 (Dr R.V. McCloskey, personal communication).

ADDITIONAL ANTI-CORE GLYCOLIPID MONOCLONAL ANTIBODIES

A number of other murine and human MABs have been developed which specifically bind to various epitopes of the core glycolipid structure of bacterial endotoxin (Table 2). One, known as T88 (Chiron, Emeryville, CA, USA) was well tolerated in phase I testing in humans^{63,64} and is currently being tested in a large multicenter phase III trial. Interestingly, this MAB, in addition to its endotoxin-neutralizing effects, may also mediate the opsonization and killing of a serum-resistant Gram-negative bacilli by human serum.⁶³ Another MAB developed by Sandoz, SDZ219-800, is a chimeric human-murine MAB which is broadly cross-reactive against smooth and rough LPS.⁶⁵ This chimeric MAB blocks endotoxin activity in the Limulus assay, cytokine production by macrophages both in vitro and in vivo and prevents endotoxin-induced lethality in D-galactosamine sensitized mice. The antibody has yet to be tested in human subjects.

Other MABs are at various stages of preclinical development or have been used principally as reagents for the study of the pathophysiology of endotoxin-induced shock. Some of these MABs prevent lethality in galactosamine-treated mice and inhibit TNF production,^{66,67} in addition to inhibiting the LPS priming of human neutrophils for superoxide production.⁶⁷ A murine IgM MAB, clone 20, binds to the α -linked KDO (2-keto-3-deoxyoctulosonic acid) moiety of the core glycolipid and appears to provide protection against endotoxin-induced lethality in mice;⁶⁸ however, this functional activity has not been verified with the MAB purified from either the ascites or hybridoma fluid, a consideration that applies to the testing of all MABs.⁶⁹ Another MAB that recognized a KDO epitope, GL11, protected against heterologous LPS lethality in sensitized mice even when given after LPS challenge.⁷⁰ MABs have been described that not only inhibit LPS-induced cytokine secretion and lethal shock, but also B cell mitogenesis.⁷¹

It is possible to re-express a low yield human MAB into a high yield murine hybridoma system. A human IgM MAB (SDJ5) which reacts to the phosphate group and the fatty acid side chains of lipid A has been suc-

cessfully expressed in a murine system.⁷² The cDNA of both the heavy and light chains of this MAb was isolated and inserted into an expression vector which was then used to transfect a non-immunoglobulin-producing murine hybridoma cell line. The resulting cell line was shown to produce a functional monoclonal antibody which was exclusively human and produced in 50-fold greater amounts than the original human parental cell line.⁷³ This process should facilitate the production of large quantities of human MAb as clinical grade material.

It is possible that the concept of anti-core glycolipid MAbs in the treatment of septic shock is valid, but that the correct choice of the specific MAb has yet to be made. The availability of antibodies which would have high binding affinity that would be readily synthesized and produced economically in large quantities, and which would have opsonophagocytic as well as endotoxin-neutralizing activity would be highly desirable. It is possible that the current MAbs could be improved by modifying their binding activity, stability or isotype. In the case of murine MAbs, humanized antibodies by CDR grafting might remove potential concerns over their immunogenicity and relatively short serum half-life.⁷⁴

CRITIQUE

Despite both the directness and deceptive simplicity of the hypothesis, namely antibody directed toward a common, toxic moiety of endotoxin has therapeutic potential, the concept of anti-endotoxin antibody has been mired in controversy. In the absence of a clearly formulated and demonstrable mechanism of action it is diffi-

cult to design a clinical trial that would yield meaningful results. Yet lacking such data, several large, costly and complex clinical trials have been conducted, with the entire concept of anti-core glycolipid antibody relying on their interpretation.³⁹ Investigators have then sought to arrive at some arithmetic conclusion of the value of anti-endotoxin antibody by tallying the success of a study whose endpoint is reduction of septic mortality during Gram-negative septic shock with the failure of another study whose endpoint is reduction in acquisition of infection.

The criticisms of both clinical and preclinical studies with core glycolipid antiserum have focused on (1) the lack of reproducibility of the protective effect, (2) the paucity of convincing data to demonstrate anti-core antibody to be protective, either in animal models or in clinical studies, particularly since the protean biological manifestations of LPS include the induction of non-antibody moieties capable of inactivating LPS,⁶ and (3) the inability of anti-core glycolipid antibodies to bind to endotoxin of smooth, bacteremic strains.

Lack of reproducibility of protective effect

It is now clear that an anti-endotoxin antiserum may function by at least 3 different mechanisms: direct neutralization of the biological activity of the LPS,^{67,71} in a manner similar to polymyxin B; promotion of the clearance of the LPS from the circulation,⁵⁶ or mediation of the opsonophagocytic killing of the bacteria.⁶³ While it is possible to assess the ability of an anti-endotoxin antiserum or MAb to neutralize LPS or promote opsonophagocytosis in vitro, it is necessary to have an animal model to assess the clearance-promoting activity of an anti-endotoxin antibody. Initially, protection from

Table 2. Activity of other anti-core glycolipid monoclonal antibodies

Monoclonal antibody	Source	Isotype	Epitope	In vitro activity	In vivo activity	Reference
Clone 20	Murine	IgM	KDO from Re	Binding to smooth LPS	Protects mice from <i>E. coli</i> challenge	Appelmek ⁶⁸
D6B-3	Murine	IgG	J5 core glycolipid	Inhibits TNF production	Inhibits TNF; protects mice from <i>E. coli</i>	Vacheron ⁶⁶
8-2/26-20	Murine	IgM	Lipid A	Inhibits LPS priming of neutrophils	Inhibits TNF; protects mice from LPS injection	Cornelissen ⁶⁷
F88	Human	IgM	Lipid A	Binds to LPS, promotes opsonization bacteriocidal effect	-	Winkelhake ⁶³
MLA-1	Murine	IgM	Lipid A from Re	Inhibits IL-1, TNF; B cell mitogenesis	Protects mice from lipid A	Ramachandra ⁷¹
SDZ 219-800	Chimeric human-mouse	IgG1 IgG2a	Core glycolipid	Inhibits Limulus reaction, TNF; IL-6 generation	Blocks rabbit pyrogen, LPS lethality in mice	Di Padova ⁶⁶
SDJ5 1.17.15	Human	IgM	Phosphate-fatty acid of lipid A	Binding to smooth LPS	-	Kazemi ⁷²
GL11	Murine	IgG2b	KDO from Re	Binds to Re LPS	Protects mice from LPS challenge	Nys ⁷⁰

the dermal Shwartzman reaction by J5 antiserum was not correlated with enhanced clearance of endotoxin from the circulation (as was previously observed with the induction of endotoxin tolerance);⁷⁵ however, the beneficial effect of J5 antiserum during infection was associated with an accelerated clearance of bacteria from the circulation.²² Consequently, from the outset, it was not clear whether the mechanism of J5 activity was through an antitoxic or opsonic effect.

Presently, there are no adequate, widely-accepted animal models that reflect the septic process in humans. As pointed out by Ziegler,⁶ in many models the dose-response curves between 100% survival and 100% death are quite steep (often occurring over a one log range of bacterial or LPS challenge), thereby making it difficult to show a reproducible protective effect from one experiment to another.

There are additional considerations with animal models that make it difficult to compare study results. Models that rely on the intravenous infusion of endotoxin or very high levels of live bacteria to initiate a septic response are able to demonstrate the acute, physiologic (primarily hemodynamic) effects of endotoxemia; however, since the endotoxin and bacteria are rapidly removed by the reticuloendothelial system, the systemic reaction to LPS ends quickly. In this and other models where the time from challenge to death is quite short, the subacute effects of systemic endotoxin, such as the multiple organ failure typical of clinical sepsis, might not have sufficient time to develop. In contrast, during clinical infection, LPS is initially found in the tissues, with a low level endotoxemia occurring secondarily over time.¹⁹ Also, with the infusion of large inocula it is difficult to infuse a sufficient excess of antibody to determine if the reagent has any therapeutic potential. Thus, it would be difficult to extrapolate the activity of an anti-glycolipid antibody in these animal models to clinical trials in patients where, given the relatively low levels of circulating bacteria or endotoxin, administering such an excess of antibody is possible. Additionally, models that need to compromise the animal host defenses in order to enhance susceptibility to infection may also alter an element necessary for anti-glycolipid antibody activity, or may obscure a mechanism by which the antibody might function in the absence of such manipulation. Finally, models in which the virulence of the bacteria is artificially enhanced (e.g. by the addition of the highly sialylated hog mucin or the addition of hemoglobin that binds nitric oxide), may place a demand on an antibody that it might not confront in clinical sepsis. In summary, a consistent benefit in both acute toxicity and subacute infection models would provide the most compelling preclinical evidence of an antibody's therapeutic potential in septic patients.

Antibody

While studies of active and passive immunization with a rough LPS mutant vaccine have shown protective efficacy in experimental and clinical studies, it has been

difficult to define immunoglobulin as the protective element.^{28,29,76} For example, post-J5 immunization serum, with a 3- to 5-fold increase in anti-J5 antibody, showed a beneficial clinical effect despite barely measurable changes in anti-J5 antibody levels in recipients.²⁸ Survival in these studies was better correlated with the receipt of post-immunization serum than with the actual level of antibody.^{28,29,76} This may reflect a technical difficulty in the antibody assays, a protective epitope other than the J5 or Re LPS antigens used in the assays or that protection is due to a non-immunoglobulin fraction in the antiserum.

Both retrospective serological surveys⁷⁷⁻⁷⁹ and experimental studies^{31,76,80,81} have attempted to correlate antibody to core glycolipid and survival. Among 175 patients with Gram-negative bacteremia, the incidence of shock and death were reduced by one-third among patients with indirect hemagglutinating (predominantly IgM) antibody titer to *Salmonella* Re LPS of $\geq 1:80$ at the onset of bacteremia.⁷⁷ This was independent of any contribution of O-specific IgG antibody which was also associated with a reduction in complications of Gram-negative bacteremia.⁷⁸ When examined by an ELISA, the presence of high levels of circulating antibody to the endotoxin core of *E. coli* J5 and to O antigen were each correlated with improved outcome in *Pseudomonas aeruginosa* septicemia.⁷⁹ While this assay could detect IgG isotype, multivariate analysis found that the IgM isotype correlated better with decreased mortality than did IgG.

More recent studies provide convincing evidence that the antibody to the core glycolipid in immune antisera could provide protection against heterologous bacterial challenge. Immunization of humans and rabbits with whole cell *Salmonella minnesota* R595 vaccines protected mice from lethal heterologous bacterial and endotoxin challenge upon passive transfer.^{76,80} Fractionation of post-immunization *S. minnesota* R595 immunization sera by sizing chromatography revealed that the protective activity in mice correlated solely with the IgM fraction.⁸⁰ Even though absorption of the antisera with the *S. minnesota* LPS removed most of the protective activity, thereby suggesting that anti-Re antibody provided the protection, measurement of antibody levels in the different aliquots of serum did not correlate with level of protection.⁷⁶ These studies would suggest that commercially prepared human IgG with high titers to core glycolipid would have little clinical utility and would also offer a partial explanation for why J5 IVIG was ineffective.³⁷

In contrast, fractionation of lapine anti-J5 antisera into IgG, IgM and non-immunoglobulin components demonstrated that both IgG and IgM isotypes mediated protection against lethal *Pseudomonas* bacteremia in a neutropenic rat model of infection. Non-immunoglobulin fractions also provided mild protection. Thus, unfractionated J5 antisera has multiple components that may affect survival and this complicates any simple interpretation of the protective effect of whole serum. Optimal protection was obtained from IgG that was eluted

from a J5 LPS affinity column (devoid of lipid A), while minimal protection was observed with the affinity column 'pass-through' enriched in anti-lipid A.⁸² Thus, these studies show that immunoglobulin fractions from post-immunization sera can mediate protection, and this protection is significantly diminished by removing immunoglobulin to either Re⁸⁰ or J5 LPS.⁸²

Administration of endotoxin induces acute phase reactants (e.g. LPS binding protein, lipoproteins, soluble CD14), cytokines and perhaps other moieties that may alter LPS activity. Warren and colleagues found non-antibody moieties of human plasma capable of neutralizing the Limulus reactivity of LPS as well, if not better, than anti-endotoxin antibody.⁸³ The above studies^{80,82} were performed under conditions in which the contribution of these variables is less likely (e.g. harvesting serum months after immunization). Similarly, while contamination of serum with LPS may induce a state of tolerance upon passive administration in immunoprophylactic studies, the protective activity that follows administration of immunoglobulin fractions with < 100 pg/ml LPS as therapy to animals already bacteremic and with circulating endotoxin levels in the nanogram range is unlikely to function through the induction of a tolerant state. Finally, some have postulated that protective activity is due not to broadly protective anti-glycolipid antibody but rather to the well known polyclonal antibody response following endotoxin administration;^{77,84} however, as noted by others, the 2- to 3-fold increase in polyclonal O antibody in many studies appears too modest to account for the protective activity.³⁹

If subsequent data confirm that anti-core glycolipid antibody mediates protection from septic complications of Gram-negative bacteremia, then it may be advantageous to develop vaccines to induce high affinity antibodies against specific core LPS epitopes and to avoid lot-to-lot variations in antibody obtained from screened plasma.⁸³ Following natural exposure, the human antibody response to core glycolipid antigens is modest compared to O antibody responses,^{85,86} and the affinity of naturally acquired anti-glycolipid antibody is also believed to be low. Consequently, anti-endotoxin antibody preparations derived from screened plasma may not be as effective as antibody obtained following immunization. If safe, effective anti-core glycolipid vaccines are to be developed, it is necessary to identify both the antibody isotype(s) and specific epitopes that provide optimal protection.

Epitope

While considerable data show a highly conserved core structure of LPS to which an anti-glycolipid antibody might be directed, it is yet unclear if there is a *specific* core epitope such that antibodies to it are more cross-protective than would be antibodies to other core epitopes. Experimental studies described above achieved highly significant protection in animal models with antibodies directed against both Re^{31,80} and J5 LPS.⁸²

Similarly, serological surveys of bacteremic patients correlated level of anti-J5⁷⁹ and anti-Re⁷⁷ antibody at the onset of infection with survival. Thus, there are data to support the efficacy of antibody to both core LPS epitopes. In contrast, there is little clinical or experimental data to support lipid A as a target for cross-protective antibody.^{26,27,39,87} In view of the lack of efficacy of anti-lipid A polyclonal antibody in these studies, it is noteworthy that considerable effort is expended in generating anti-endotoxin MAbs directed toward the lipid A component.

There is evidence that the J5 core epitope is a distinct core structure not found in *S. minnesota* R595.⁸⁸ Structural studies on the core of *Salmonella* have identified an epitope on intact laboratory strains of bacteria with a smooth LPS phenotype that is accessible to anti-core antibody of the Ra through Rc (but not Rd and Re) chemotypes.⁸⁹ Interestingly, this epitope is not accessible on strains cultured from human blood. Further, elicitation of antibody to J5 epitope(s) occurs in the absence of antibody response to Re or lipid A epitopes.^{90,91}

Binding of anti-endotoxin antibody to smooth LPS

It is difficult to demonstrate the binding of anti-endotoxin antibodies to smooth LPS in conventional ELISA or Western blot types of analysis. This might be due to the physical orientation or presentation of the LPS, an amphipathic molecule with poor solubility, whereby critical epitopes may be selectively masked or exposed by the micellar formation of LPS. There is also a tendency for immunoglobulin to stick non-specifically to hydrophobic structures such as LPS. Fluid-phase methods designed to assess such binding have been developed that may overcome these barriers.^{53,54,92} Thus as bacteria grow in broth, rate nephelometry assays can detect binding of anti-endotoxin antibody (HA-1A) to dividing bacteria, and this is inhibited by preincubation of the MAb with lipid A.⁹² Pretreatment of smooth bacteria with inhibitory concentrations of antibiotics has been shown to expose core LPS epitopes to antibody binding.⁵⁴ Thus, it may be possible to better evaluate the binding of potentially useful antibodies, and to do so in a relevant manner, i.e. binding to bacteria rather than to purified LPS.

In addition to the above-mentioned problems, there are two additional considerations each of which might affect the lack of reproducibility of the data: the preparation of vaccine; and the immunization regimen.

Vaccine preparation

While some investigators have focused on the source of the J5 isolate used for preparation of the antiserum,⁹³ relatively little attention has been given to vaccine preparation. For their studies, Braude and colleagues obtained an isolate of an Rc chemotype mutant (J5) of *E. coli* O111:B4 from Elbein & Heath; however, they fur-

ther selected a stable, rough mutant that, unlike the original J5 strain, did not incorporate galactose when added exogenously to the culture. From this behavior it was inferred, but not formally demonstrated, that the Braude J5 mutant had a second mutation.⁹⁴ There are no 'seed lots' of this strain in a repository, such as in the American Type Culture Collection.

Regardless of the source of J5 isolate, culture of this 'leaky' mutation results in colony forming units that vary in degree of roughness. ('Leakiness' refers to the possibility that a genetic mutation results in a reduction, but not complete inactivation, of an enzymatic activity, or that the phenotypic expression of the enzymatic defect is variably expressed in a population and depends on the occurrence of a secondary mutation.) Hence within a 'pure' J5 culture, it is necessary to select a colony with a rough phenotype to insure a vaccine that elicits anti-core glycolipid antibody. In the original preparation of the O113 vaccine, serial passage was concluded by exposure of the culture to anti-smooth parental antisera to insure only the presence of rough mutants in the vaccine.²⁰

Immunization regimens

Initial studies with rough mutant immunization elicited protective activity with regimens that generated IgM (19S) and IgG (7S) antibody, as well as with hyperimmunization regimens.^{20,81} Despite the effectiveness of all 3 regimens, subsequent studies were conducted with serum collected at the height of the hemagglutinating (IgM) antibody response, with little explanation for this choice. Subsequently, there have been few attempts to optimize immunization regimens, perhaps since some experimental data, particularly those of McCabe et al with Re mutants of *Salmonella*, suggested the importance of the IgM isotype.⁷⁷ If one were to desire an IgG isotype anti-core glycolipid antibody, perhaps for preparation of an enriched IVIG, one might choose to harvest plasma at a later time point, perhaps after one or two booster doses.

Comparison of the protective activity from serum collected from human volunteers immunized with different doses of Re mutant vaccine and at variable frequencies revealed that regardless of primary immunization schedule, protective activity progressively increased until 6 weeks after immunization, independent of measured antibody levels.⁷⁶ In this study, no increase in antibody titers could be shown following booster immunization, but the protective activity following these booster doses was not assessed. In earlier studies, animals that received an intensive immunization regimen with *S. minnesota* Re LPS (up to 11 doses over 2 months) developed highly protective levels of antibody.³¹ We found that anti-core glycolipid antibody harvested after booster immunization may have better activity than antibody harvested after a primary series.⁸² Of note, Dale and colleagues reported that following 3 consecutive daily injections of J5 vaccine to a human volunteer, there was a 10-fold increase in IgG

anti-J5 antibody that peaked at 9 months, and this IgG was bactericidal for a serum-resistant strain of gonococcus.⁹⁵

GENERAL ISSUES IN THE DEVELOPMENT OF ANTI-CORE GLYCOLIPID ANTIBODY

Clinical design

The 1982 J5 antisera clinical study identified groups of patients likely to benefit from adjuvant anti-endotoxin therapy: those with severe sepsis and Gram-negative bacteremia, and those with septic shock requiring vasopressors for > 72 h. These were essentially the same groups that were identified on retrospective analysis to have derived the greatest benefit from treatment with HA-1A MAb,⁴⁹ anti-TNF MAb⁹⁶ and IL-1ra.⁹⁷ Had these groups been selected as the primary target population rather than the entire septic group in an intent-to-treat analysis, it is conceivable that a significant treatment effect could have been demonstrated with some of these agents. In addition to the choice of primary target population, studies were often terminated when there was barely sufficient numbers of patients to provide adequate statistical power to each study. This created a situation where the loss of a few patients from one treatment group or another would obviate the treatment effect and put the entire study result into question. While premature termination may have been dictated by the status of competing studies, in the end it was counterproductive.

In retrospect, it also appears that many antiseptis products were hastened into clinical trials before an adequate scientific record, preferably published but even unpublished, was established for each of the MAbs, perhaps with the hope that the demonstration of clinical efficacy could bypass the need for strong preclinical scientific data. Certainly, the availability of supporting preclinical data would have helped in the presentations to the FDA Advisory Panel, which was composed predominantly of members of the academic community. Since a large volume of studies were published *after* the Panel meetings,^{50,54,56,57,67,92} the lack of scientific evidence was not due to daunting scientific barriers. Moreover, the availability of strong scientific data would have helped physicians in their recommendations to their hospitals that these expensive agents, with their significant impact on hospital costs, be placed on the formulary. In summary, economic and patent issues appeared to have taken precedence over scientific issues, ultimately to the detriment of anti-endotoxin antibody development.

Regulatory issues

Several regulatory issues were raised during consideration of recent trials of antiseptis products which may place too severe a test for the approval of potentially useful reagents. First, it is useful to consider that the se-

lection of discriminatory patient populations for determining the efficacy of an anti-sepsis drug in a clinical trial may differ from the identification of the more heterogeneous population of patients who may eventually derive benefit from the drug once approved. For determining the efficacy of a drug in early clinical trials, it might be important to study only those patients with potentially reversible physiology in whom the effect of a treatment can be measured. The inclusion of those with irreversible physiologic changes not amenable to modification by the drug might not be appropriate for the purpose of determining whether the drug has efficacy and might mask a clinical effect from the drug. Second, the use of (28-day) all-cause mortality as the primary endpoint to measure the efficacy of a product instead of an improvement in sepsis-associated physiology also might mitigate against approval of a potentially useful drug. Anti-endotoxin agents can only be expected to diminish the risk of mortality attributable to endotoxin-induced injury and not be expected to have a generic capacity to alter physiologic damage due to underlying disease. Requiring such agents to reduce all-cause mortality in this severely ill population may be overly stringent and not correlate with expectations in clinical practice. By analogy, the primary endpoint for trials of antibiotic therapy usually is cure of infection rather than mortality. Similarly, antihypertensive or diabetes therapy trials also use improvement in physiology rather than mortality endpoints. Since the time of death of a patient on life support is often "negotiated" between family and patient, mortality, particularly at a specific time, is not an unequivocal endpoint. Even the use of attributable, as opposed to all-cause, mortality as an endpoint may be undesirable since this endpoint would require an even greater number of subjects than those entered into these recent trials.

Third, the need for each anti-endotoxin agent to be efficacious when used alone may also be an unrealistic requirement. Sepsis is a progressive pathophysiologic process which has multiple stages. Initially, there is often a bacteremic phase during which time the administration of specific antibodies (anti-O or capsular) may hasten bacterial clearance and minimize later septic complications. After this stage, endotoxin, liberated either by growing bacteria or by treatment with antibiotics, may circulate until bound by an immune-reactive target cell (e.g. macrophage, endothelial cell). Anti-endotoxin antibodies may work optimally at this phase of the process to either neutralize the biologic activity of lipid A or to promote its clearance before initiating an inflammatory cascade. If the biologically active endotoxin cannot be intercepted before interacting with these immune reactive cells, several host inflammatory mediator cascades might be activated that result in clinical sepsis. Therapeutic agents for sepsis, such as anti-TNF MAb or IL-1ra, are designed to intervene at this, but not earlier stages. In fact, there is evidence that anticytokines may be detrimental if given in the early phases of infection.⁹⁸ Once circulating endotoxin has initiated a cytokine cascade, it is unlikely that treatments, such as

anti-endotoxin antibodies, will reverse that cascade. Similarly, unless infection, a continuing source of endotoxin, is treated with appropriate antibiotics, it is also unlikely for agents directed at the later cytokine cascade (anti-TNF MAb or IL-1ra) would have much impact. Thus, it may be unduly optimistic to expect a product aimed at only one step in this process to show significant efficacy for all patients who may appear anywhere along this continuum of the septic process. Ideally, combinations of treatments directed at sequential steps of the septic process may be a more rational strategy, as has been demonstrated experimentally.⁹⁹ Antisepsis therapy may be analogous to combination cancer chemotherapy regimens where single agents are not sufficiently active alone to be effective but combination therapy may be highly effective. Finally, should combination immunotherapy be optimal for the adjunctive therapy of sepsis, then it is incumbent on manufacturers to insure that the individual components of that treatment are cost effective. Adjuvant therapy will need to either save total health care resources by shortening length of stay in special care units, or be highly effective in saving lives (preferably both) in order to be approved for use in clinical medicine. This will be a formidable challenge.

While recent clinical trials with anti-endotoxin MAbs were disappointing, they have focused considerable critical thought on the concept of anti-endotoxin antibody and generated new experimental approaches. This experience and a greatly expanded database could significantly hasten the development of effective reagents with which to treat or prevent sepsis.

References

1. Ziegler E J, Fisher C J Jr, Sprung C L et al. Treatment of gram-negative bacteremia and septic shock with HA-1A human monoclonal antibody against endotoxin. *N Engl J Med* 1991; 324: 429-436.
2. Greenman R L, Schein R M H, Martin M A et al. A controlled clinical trial of E5 murine monoclonal IgM antibody to endotoxin in the treatment of gram-negative sepsis. *JAMA* 1991; 266: 1097-1102.
3. McCloskey R V. HA-1A efficacy and septic shock (CHES) Trial results. The 3rd Annual Meeting on Advances in the Diagnosis, Prevention and Treatment of Endotoxemia and Sepsis. (International Business Communications), Philadelphia, 18 June, 1993: 119-123.
4. Wenzel R, Bone R, Fein A et al. Results of a second double-blind, randomized, controlled trial of antiendotoxin antibody E5 in gram-negative sepsis. Program and Abstracts of the 31st International Conference on Antimicrobial Agents and Chemotherapy. 1991; 294 (abstr 1170).
5. Baumgartner J D, Heumann D, Gerain J, Weinbreck P, Grau G E, Glauser M P. Association between protective efficacy of anti-lipopolysaccharide (LPS) antibodies and suppression of LPS-induced tumor necrosis factor alpha and interleukin 6. Comparison of O side chain-specific antibodies and core LPS antibodies. *J Exp Med* 1990; 171: 889-896.
6. Ziegler E J. Protective antibody to endotoxin core: the emperor's new clothes? *J Infect Dis* 1988; 158: 286-290.
7. Favorte G O, Morgan H R. Effects produced by intravenous injection in man of a toxic antipenic material derived from *Escherichia*

- typhosa: clinical, hematological, chemical and serological studies. *J Clin Invest* 1942; 21: 589-595.
8. Felty A R, Keefer C S. Bacillus coli sepsis. A clinical study of twenty-eight cases of blood stream infection by the colon bacillus. *JAMA* 1924; 82: 1430-1433.
 9. Waisbren B A. Bacteremia due to gram-negative bacilli other than the *Salmonella*. *Arch Intern Med* 1951; 88: 467-488.
 10. Braude A I, Siemiaske J, Williams D, Sanford J P. Overwhelming bacteremic shock produced by Gram-negative bacilli: a report of four cases with one recovery. *Univ Michigan Med Bull* 1953; 19: 23-42.
 11. Finland M, Jones W F Jr, Barnes M W. Occurrence of serious bacterial infections since introduction of antibacterial agents. *JAMA* 1959; 170: 2188-2197.
 12. Rogers D E. The changing pattern of life-threatening microbial disease. *N Engl J Med* 1959; 261: 677-683.
 13. Sjostedt S. Pathogenicity of certain serological types of *E. coli*. Their mouse toxicity, hemolytic power, capacity for skin necrosis and resistance to phagocytosis and bactericidal faculties of human blood. *Acta Pathol Microbiol Scand* 1946; suppl 63: 1-148.
 14. Orskov I, Orskov E. *Escherichia coli* in extra-intestinal infections. *J Hyg* 1985; 95: 551-575.
 15. Roantree R J, Pappas N C. The survival of strains of enteric bacilli in the blood stream as related to their sensitivity to the bactericidal effect of serum. *J Clin Invest* 1960; 39: 82-88.
 16. Luderitz O, Galanos C, Risse H J et al. Structural relationships of *Salmonella* O and R antigens. *Ann N Y Acad Sci* 1966; 133: 349-374.
 17. Subbaran V, Stocker B A D. Rough mutants of *Salmonella typhimurium*. *Genetics*. *Nature* 1964; 201: 1298-1299.
 18. Osborn M J. Biosynthesis and structure of the core region of the lipopolysaccharide in *Salmonella typhimurium*. *Ann N Y Acad Sci* 1966; 133: 375-383.
 19. Braude A I, Jones J L, Douglas H. The behavior of *Escherichia coli* endotoxin (somatic antigen) during infectious arthritis. *J Immunol* 1963; 90: 297-312.
 20. Tate W J, Douglas H, Braude A I. Protection against lethality of *E. coli* endotoxin with 'O' antiserum. *Ann N Y Acad Sci* 1966; 133: 746-762.
 21. Braude A I, Ziegler E J, McCutchan J A, Douglas H. Immunization against nosocomial infection. *Am J Med* 1981; 70: 463-466.
 22. Ziegler E J, Douglas H, Sherman J E, Davis C E, Braude A I. Treatment of *E. coli* and *Klebsiella* bacteremia in agranulocytic animals with antiserum to a UDP-gal epimerase-deficient mutant. *J Immunol* 1973; 111: 433-438.
 23. Ziegler E J, McCutchan J A, Douglas H, Braude A I. Prevention of lethal *Pseudomonas* bacteremia with epimerase-deficient *E. coli* antiserum. *Trans Assoc Am Physicians* 1975; 81: 101-108.
 24. Chedid I, Parant M, Parant F, Boyer F. A proposed mechanism for natural immunity to enterobacterial pathogens. *J Immunol* 1968; 100: 292-301.
 25. McCabe W R. Immunization with R mutants of *S. minnesota*. I. Protection against challenge with heterologous Gram-negative bacilli. *J Immunol* 1972; 108: 601-610.
 26. Johns M, Skehill A, McCabe W R. Immunization with rough mutants of *Salmonella minnesota*. IV. Protection by antisera to O and rough antigens against endotoxin. *J Infect Dis* 1983; 147: 57-67.
 27. Bruins S C, Stumacher R, Johns M A, McCabe W R. Immunization with R mutants of *Salmonella minnesota*. III. Comparison of the protective effect of immunization with lipid A and the R mutant. *Infect Immun* 1977; 17: 16-20.
 28. Ziegler E J, McCutchan J A, Fierer J et al. Treatment of gram-negative bacteremia and shock with human antiserum to a mutant *Escherichia coli*. *N Engl J Med* 1982; 307: 1225-1230.
 29. Baumgartner J D, Glauser M P, McCutchan J A et al. Prevention of gram-negative shock and death in surgical patients by antibody to endotoxin core glycolipid. *Lancet* 1985; 2: 59-63.
 30. McCutchan J A, Wolf J L, Ziegler E J, Braude A I. Ineffectiveness of single-dose human antiserum to core glycolipid (*E. coli* J5) for prophylaxis of bacteremic, gram-negative infections in patients with prolonged neutropenia. *Schweiz Med Wochenschr* 1983; 113 suppl 14: 40-45.
 31. Young L S, Stevens P, Ingram J. Functional role of antibody against 'core' glycolipid of *Enterobacteriaceae*. *J Clin Invest* 1975; 56: 850-861.
 32. De Simone C, Delogu G, Corbetta G. Intravenous immunoglobulins in association with antibiotics: a therapeutic trial in septic intensive care unit patients. *Crit Care Med* 1988; 16: 23-26.
 33. Schedel I, Dreikhausen U, Nentwig B et al. Treatment of gram-negative septic shock with an immunoglobulin preparation: a prospective, randomized clinical trial. *Crit Care Med* 1991; 19: 1104-1113.
 34. Lachman E, Pitsoe S B, Gaffin S L. Anti-lipopolysaccharide immunotherapy in the management of septic shock of obstetrical and gynecological origin. *Lancet* 1984; 1: 981-983.
 35. Cometta A, Baumgartner J-D, Lee M L, Hanique G, Glauser M-P, IVIG Collaborative Study Group. Prophylactic intravenous administration of standard immune globulin vs compared with core lipopolysaccharide immune globulin in patients at high risk of postsurgical infection. *N Engl J Med* 1992; 327: 234-240.
 36. Fomsgaard A, Baek L, Fomsgaard J S, Engquist A. Preliminary study on treatment of septic shock patients with anti-lipopolysaccharide IgG from blood donors. *Scand J Infect Dis* 1989; 21: 697-708.
 37. Calandra T, Glauser M P, Schellekens J, Verhoef J, Swiss-Dutch J5 Immunoglobulin Study Group. Treatment of Gram-negative septic shock with human IgG antibody to *Escherichia coli* J5: a prospective, double-blind, randomized trial. *J Infect Dis* 1988; 158: 312-319.
 38. J5 Study Group. Treatment of severe infectious purpura in children with human plasma from donors immunized with *Escherichia coli* J5: a prospective double-blind study. *J Infect Dis* 1992; 165: 695-701.
 39. Baumgardner J-D. Immunotherapy with antibodies to core lipopolysaccharide: a critical appraisal. *Infect Dis Clin North Am* 1991; 5: 915-927.
 40. Warren H S, Novitsky T J, Bucklin A, Kania S A, Siber G R. Endotoxin neutralization with rabbit antisera to *Escherichia coli* J5 and other Gram-negative bacteria. *Infect Immun* 1987; 55: 1668-1673.
 41. Greisman S E, DuBuy J B, Woodward C L. Experimental Gram-negative bacterial sepsis: reevaluation of the ability of rough mutant antisera to protect mice. *Proc Soc Exp Biol Med* 1987; 158: 482-490.
 42. Warren H S, Danner R L, Munford R S. Anti-endotoxin monoclonal antibodies. *N Engl J Med* 1992; 326: 1153-1156.
 43. Bone R C. A critical evaluation of new agents for the treatment of sepsis. *JAMA* 1991; 266: 1683-1691.
 44. Young L S, Gascon R, Alam S, Bermudez L E. Monoclonal antibodies for treatment of Gram-negative infections. *Rev Infect Dis* 1989; 11: S1564-S1571.
 45. Teng N N H, Kaplan H S, Hebert J M et al. Protection against Gram-negative bacteremia and endotoxemia with human monoclonal IgM antibodies. *Proc Natl Acad Sci U S A* 1985; 82: 1790-1794.
 46. Whiller A P, Hardie W D, Bernard G. Studies of an antiendotoxin antibody in preventing the physiologic changes of endotoxemia in awake sheep. *Am Rev Respir Dis* 1990; 142: 775-780.
 47. Romulo R L C, Palardy J E, Opal S M. Efficacy of anti-endotoxin monoclonal antibody J5 alone or in combination with ciprofloxacin in neutropenic rats with *Pseudomonas sepsis*. *J Infect Dis* 1993; 167: 126-130.
 48. Ziegler E J, Teng N N H, Douglas H, Wunderlich A, Berger H J, Bolmer S. Treatment of *Pseudomonas* bacteremia in neutropenic rabbits with human monoclonal IgM antibody against *E. coli* lipid A. *Clin Res* 1987; 35: 619A.
 49. Ziegler E J, Fisher C J Jr, Sprung C L et al, and the HIA-1A Sepsis Study Group. Treatment of Gram-negative bacteremia in septic shock with HIA-1A human monoclonal antibody against endotoxin: a randomized, double-blind, placebo-controlled trial. *N Engl J Med* 1991; 325: 429-436.

50. Fujihara Y, Bogard W C, Lei M G, Daddona P E, Morrison D C. Monoclonal anti-lipid A IgM antibodies HA-1A and E5 recognize distinct epitopes on lipopolysaccharide and lipid A. *J Infect Dis* 1993; 168: 1429-1435.
51. Bogard W C Jr, Siegel S A. The human monoclonal antibody HA-1A: studies on the epitope location within the endotoxin molecule and epitope exposure on the surface of viable Gram-negative bacteria. *Circ Shock* 1991; 34: 119 (abstract).
52. Parent J B, Gazzano Santoro H, Wood D M et al. Reactivity of monoclonal E5 with endotoxin. II. Binding to short and long-chain smooth lipopolysaccharides. *Circ Shock* 1992; 38: 67-73.
53. Warren H S, Amato S F, Fitting C et al. Assessment of ability of murine and human anti-lipid A monoclonal antibodies to bind and neutralize lipopolysaccharide. *J Exp Med* 1993; 177: 89-97.
54. Siegel S A, Evans M E, Pollack M et al. Antibiotics enhance binding by human lipid A reactive monoclonal antibody HA-1A to smooth, Gram-negative bacteria. *Infect Immun* 1993; 61: 512-519.
55. Quezada Z M N, Natanson C, Alling D W et al. A controlled trial of HA-1A in a canine model of Gram-negative septic shock. *JAMA* 1993; 269: 2221-2227.
56. Krieger J J, Fletcher R C, Siegel S A et al. Human anti-endotoxin antibody HA-1A mediates complement-dependent binding of *Escherichia coli* J5 lipopolysaccharide to complement receptor type 1 of human erythrocytes and neutrophils. *J Infect Dis* 1993; 167: 865-875.
57. Wortel C H, von der Molen M A M, van Devender S J H et al. Effectiveness of a human monoclonal anti-endotoxin antibody (HA-1A) in Gram-negative sepsis: relationship to endotoxin and cytokine levels. *J Infect Dis* 1992; 166: 1367-1374.
58. Wenzel R P. Anti-endotoxin monoclonal antibodies - a second look. *N Engl J Med* 1992; 326: 1151-1153.
59. Schulman K A, Gillick H A, Rubin H, Eisenberg J M. Cost effectiveness of HA-1A monoclonal antibody for Gram-negative sepsis. *JAMA* 1991; 266: 3466-3471.
60. Baumgartner J D. Monoclonal anti-endotoxin antibodies for the treatment of Gram-negative bacteremia and shock. *Eur J Clin Microbiol Infect Dis* 1990; 9: 711-716.
61. Tanio C P, Feldman H I. The HA-1A monoclonal antibody for Gram-negative sepsis. *N Engl J Med* 1991; 325: 280.
62. Wedel N I. Clinical experience with antiendotoxin monoclonal antibody E5 in the treatment of Gram-negative sepsis. The 2nd Annual Meeting on Advances in the Diagnosis, Prevention and Treatment of Endotoxemia and Sepsis. (International Business Communications) 22 June 1992: 20-37.
63. Winkelhake J L, Gauny S S, Senyk G, Piazza D, Stevens P. Human monoclonal antibodies to glycolipid A that exhibit complement species-specific effector functions. *J Infect Dis* 1992; 165: 26-33.
64. Daifuku R, Haentfling K, Young J, Groves E S, Turrell C, Meyers F J. Phase I study of antilipopolysaccharide human monoclonal antibody MAB-T88. *Antimicrob Agents Chemother* 1992; 36: 2349-2351.
65. DiPadova F E, Barclay R, Brade H et al. SDZ 219-800: a chimeric broadly cross-reactive and cross-neutralizing anti-core LPS antibody. *Circ Shock* 1993; 1(suppl): 47 (abstr 12.3).
66. Vacheron F, Mandine E, Lenaour R, Smets P, Zalis Z, Guenounou M. Inhibition of production of tumor necrosis factor by monoclonal antibodies to lipopolysaccharides. *J Infect Dis* 1992; 165: 873-878.
67. Cornelissen J J, Makel I, Algra A et al. Protection against lethal endotoxemia by anti-lipid A murine monoclonal antibodies: comparison of efficacy with that of human anti-lipid A monoclonal antibody HA-1A. *J Infect Dis* 1993; 167: 876-881.
68. Appelmeik B J, Verweij-VanVought A M J J, Maaskant J J, Schouten W F, Thijs L G, MacLaren D M. Monoclonal antibodies detecting novel structures in the core region of *Salmonella minnesota* lipopolysaccharide. *FEMS Microbiol Lett* 1987; 40: 71-74.
69. Silva A T, Appelmeik B J, Cohen J. Purified monoclonal antibody to endotoxin core fails to protect mice from experimental Gram-negative sepsis. *J Infect Dis* 1993; 168: 256-257.
70. Nys M, Cloes J M, Demonty J, Jansin L. Protective effects of polyclonal sera and of monoclonal antibodies active to *Salmonella minnesota* Re595 lipopolysaccharide during experimental endotoxemia. *J Infect Dis* 1990; 162: 1087-1095.
71. Ramachandra R N, Berczi A, Sehon A H, Berczi I. Inhibition of lipid A- and lipopolysaccharide-induced cytokine secretion, B cell mitogenesis, and lethal shock by lipid A specific murine monoclonal antibodies. *J Infect Dis* 1993; 167: 1151-1159.
72. Kazemi M, Huntentburg C C, Bubbers J E. Lipopolysaccharide epitope specificity and binding cross-reactivity of the human IgM anti-lipid A monoclonal antibody SDJ5-1.17.15. *Mol Immunol* 1994 (in press).
73. Dorai H, Bubbers J E, Gillies S D. Cloning and reexpression of a functional human IgM anti-lipid A antibody. *Hybridoma* 1992; 11: 667-675.
74. Mayforth R D, Quintans J. Designer and catalytic antibodies. *N Engl J Med* 1990; 323: 173-178.
75. Braude A I, Douglas H. Passive immunization against the local Schwartzman reaction. *J Immunol* 1972; 108: 505-512.
76. DeMaria A Jr, Johns M A, Berberich H, McCabe. Immunization with rough mutants of *Salmonella minnesota*: initial studies in human subjects. *J Infect Dis* 1988; 158: 301-311.
77. McCabe W R, Kreger B E, Johns M. Type-specific and cross-reactive antibodies in Gram-negative bacteremia. *N Engl J Med* 1972; 287: 261-267.
78. Zinner S H, McCabe W R. Effects of IgM and IgG antibody in patients with bacteremia due to gram-negative bacilli. *J Infect Dis* 1976; 133: 37-45.
79. Pollack M, Huang A I, Prescott R K et al. Enhanced survival in *Pseudomonas aeruginosa* septicemia associated with high levels of circulating antibody to *Escherichia coli* endotoxin core. *J Clin Invest* 1983; 72: 1874-1881.
80. McCabe W R, DeMaria A Jr, Berberich H, Johns M. Immunization with rough mutants of *Salmonella minnesota*: protective activity of IgM and IgG antibody to the R595 (Re chemotype) mutant. *J Infect Dis* 1988; 158: 291-300.
81. Brown K R, Douglas H, Braude A I. Prevention of death from endotoxin with antisera. II. Elimination of the risk of anaphylaxis to endotoxin. *J Immunol* 1971; 106: 324-333.
82. Bhattacharjee A K, Opal S M, Drabick J J et al. Affinity purified *E. coli* J5 LPS-specific IgG protects neutropenic rats against Gram-negative bacterial sepsis. *Clin Res* 1993; 41: 247A.
83. Warren H S, Novitsky T J, Ketcham P A, Roslansky P F, Kania S, Siber G R. Neutralization of bacterial lipopolysaccharides by human plasma. *J Clin Microbiol* 1985; 22: 590-595.
84. Siber G R, Kania S A, Warren H S. Cross-reactivity of rabbit antibodies to lipopolysaccharide of *Escherichia coli* J5 and other Gram-negative bacteria. *J Infect Dis* 1985; 152: 954-964.
85. Cross A, Sidberry H, Sadoff J C. The human antibody response during natural bacteremic infection with Gram-negative bacilli against lipopolysaccharide core determinants. *J Infect Dis* 1989; 160: 225-236.
86. Brauner A, Kallénus G, Wrangsell G, Wretling B, Svenson S B. Antibody responses to *Escherichia coli* J5 lipopolysaccharide and to *Salmonella* porin in patients with bacteremia. *Microb Pathog* 1986; 1: 475-481.
87. Galanos C, Luderitz O, Westphal O. Preparation and properties of antisera against lipid A component of bacterial lipopolysaccharides. *Eur J Biochem* 1971; 24: 116-122.
88. Barclay G R, Scott B B. Serological relationships between *Escherichia coli* and *Salmonella* smooth- and rough-mutant lipopolysaccharides as revealed by enzyme-linked immunosorbent assay for human immunoglobulin G antiendotoxin antibodies. *Infect Immun* 1987; 55: 2706-2714.
89. Nnalue N A, Lind S M, Lindberg A A. The disaccharide L-alpha-D-heptose 1-7-L-alpha-D-heptose 1 of the inner core domain of *Salmonella* lipopolysaccharide is accessible to antibody and is the epitope of a broadly reactive monoclonal antibody. *J Immunol* 1992; 149: 2722-2728.
90. Schwartz T A, Alcidi D V, Numsuwan V, Gocke D J. Characterization of the human antibody response to an *Escherichia coli* O111:B4(J5) vaccine. *J Infect Dis* 1988; 158: 1125-1136.

91. Baumgartner J D, Heumann D, Calandra T, Glauser M P. Antibodies to lipopolysaccharides after immunization of humans with the rough mutant *Escherichia coli* J5. *J Infect Dis* 1991; 163: 769-772.
92. Mascelli M A, Frederick B, Ely T et al. Reactivity of the human antiendotoxin immunoglobulin M monoclonal antibody HA-1A with lipopolysaccharides from rough and smooth Gram-negative organisms. *Infect Immun* 1993; 61: 1756-1763.
93. Appelmek B J, DiPadova F, van der Meer N et al. Heterogeneity of *Escherichia coli* J5 strains is related to their ability to induce cross-reactive antibodies. Program Second Conference of the International Endotoxin Society, Vienna 1992, abstr. 102.
94. Ziegler E J, McCutchan J A, Braude A I. Clinical trial of core glycolipid antibody in Gram-negative bacteremia. *Trans Assoc Am Physicians* 1978; 91: 253-258.
95. Dale P A, McQuillen D P, Gulati S, Rice P A. Human vaccination with *Escherichia coli* J5 mutant induces cross-reactive bactericidal antibody against *Neisseria gonorrhoeae* lipopolysaccharide. *J Infect Dis* 1992; 166: 316-325.
96. Wherry J, Wenzel R, Wunderink R et al, and the TNF Monoclonal Antibody Study Group. Monoclonal antibody to human tumor necrosis factor: multicenter efficacy and safety study in patients with sepsis syndrome. Program and abstracts from the 33rd Interscience Conference on Antimicrobial Agents and Chemotherapy, New Orleans, October 1984; abstr 696.
97. Fisher C J Jr, Slotman G J, Opal S M et al, and the IL-1-ra Sepsis Study Group. Initial evaluation of human recombinant interleukin-1 receptor antagonist in the treatment of the sepsis syndrome. *Crit Care Med* 1994; 22: 12-21.
98. Havell E A, Moldawer L L, Helfgott D, Kilian P L, Sehgal P B. Type 1 interleukin-1 receptor blockade exacerbates murine listeriosis. *J Immunol* 1992; 148: 1486-1491.
99. Cross A S, Opal S M, Palardy J E, Bodmer M W, Sadoff J C. The efficacy of combination immunotherapy in experimental *Pseudomonas* sepsis. *J Infect Dis* 1993; 167: 112-118.

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
--	---	--	----------------------------

NSN 7540-01-280-5500

Standard Form 298 Rev 1-89

JOURNAL OF ENDOTOXIN RESEARCH

INSTRUCTIONS TO AUTHORS

Original articles, editorial correspondence, case reports, notices and suggestions for review articles should be sent to:

David C. Morrison PhD, Associate Director, Cancer Center,
University of Kansas Medical Center, 3901 Rainbow Blvd,
Kansas City, KS 66160-7832, USA. Fax (+1) 913 588 1388.

Authors are requested to submit three copies of their typescript and illustrations.

The Editor cannot accept responsibility for damage or loss of material or disks.

A paper is accepted for publication on the understanding that it has not been submitted simultaneously to another journal in the English language.

Papers that contain the results of human and/or animal studies will only be accepted for publication if it is made clear that a high standard of ethics was applied in carrying out the investigations. In the case of invasive studies of humans, papers should include a statement that the research protocol was approved by a local ethical committee.

The Editor reserves the right to make editorial and literary corrections. Any opinions expressed or policies advocated do not necessarily reflect the opinions or policies of the Editor.

COPYRIGHT

In order for us to ensure maximum dissemination and copyright protection of material published in the Journal, copyright must be explicitly transferred from author to publisher.

The copyright transfer agreement to be used for the Journal is reproduced in the first issue. A copy of this agreement will be sent to the principal author, and must be signed by the principal author before any paper can be published.

We assure you that no limitation will be put on your personal freedom to use material contained in the paper without requesting permission, provided acknowledgement is made to the Journal as the original source of publication.

PRESENTATION OF TYPESCRIPTS

These instructions are in accordance with the International Committee of Medical Journal Editors. *Uniform requirements for manuscripts submitted to biomedical journals*. BMJ 1991; 302 (6772).

Disk submission. We encourage submission of the final version of your article on disk. We prefer 5.25" or 3.5" disks prepared on IBM compatible computers or high density disks prepared on Macintosh PCs. We also require 3 hard copies (along with tables and illustrations) of the final version of your manuscript which must be exact copies of that supplied on disk, as the manuscript may be used to set your paper if setting from disk proves impracticable.

Please ensure that all sections of your article are in a single file in the order set out below and with references in the cor-

rect style. Figures will be handled separately and should be supplied as hard copies on separate sheets; only include duplicate copies on disk if they can be supplied as EPS files. Tables, with their captions, and figure legends should be included in the main text file after the reference list.

Instructions for typing. Please read these carefully and give a copy to your typist if you are not preparing the disk yourself.

- Allow at least 3 cm margin all round.
- Set your line spacing to double spacing.
- Do not justify your text — range left allow it to run ragged on the right-hand margin. Many word processing packages have the justification mode automatically turned on and this must be turned off.
- Turn off the automatic hyphenation option. Only use hyphens when they are part of a word.
- Never use the letter 'l' for the number 1. Never use the letter 'O' for the number zero '0'.
- Never use the carriage return (enter) at the end of lines within a paragraph. Use the carriage return only at the end of paragraphs or after a heading.
- Use normal type for headings. Capitalize the first letter of the first word only. They should be ranged left and not centred. Code your headings (on the printout only) by labelling them (1) for a major heading or (2) for a minor heading.
- Do not underline headings. Do not type them in bold or italic, or type a colon or full stop after them.
- Please use single quotation marks. Only use double quotation marks for quotes within quotes.
- If your PC has italics, use sparingly. You may underline words if you want to indicate italics and this is not available on your word processor.
- Always insert a space between a number and a unit, e.g. 5 mm.
- Do not indent lists. However, if preparing a list within a list, use a tab, not a space to indent the sublist. Bullets, numbers, alphabet characters or dashes may be used but please use rationally and consistently.
- Retain a copy of the disk and the printout you send.
- Remove any backup files from the disk before submission.
- Clearly label the disk with your name, the title of the paper, and details of the hardware and software used.
- Ensure the disk is protected to avoid damage or X-ray inspection during transit.
- **Your printout must be identical to your disk as the printout may be used if setting from the disk proves impracticable.**

FULL PAPERS

Papers should be set out as follows, with each section beginning on a separate sheet: title page, summary, text, acknowledgements, references, tables, captions to illustrations.

Title page. The title page should give the following informa-