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 four studies completed on two 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. The question was rendered even more relevant given the decade-old controversy surrounding the efficacy of polyclonal antibodies to endotoxin, particularly antibody to the JS (Re chemotype) mutant of Escherichia coli O111:B4, a conceptual progenitor of the HA-1A and E-5 MAbs. 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.
Minireview

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

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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 studies completed on 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 MAb, the disappointing results raised the question whether the use of anti-endotoxin antibodies in the treatment of sepsis was still a viable concept. 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 (Re chemotype) mutant of Escherichia coli O111:B4, a conceptual progenitor of the HA-1A and E-5 MAb.

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, 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, the clinical syndrome of Gram-negative bacterial sepsis was not described until the early 1950s. Nevertheless, extra-intestinal 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 Finland and Rogers documented the ascendency 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. The discovery of protein 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. A milestone was achieved with the elucidation of the structural
features of Gram-negative bacterial lipopolysaccharide through both biochemical and bacterial genetic studies. 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 ANTICORE GLYCOLIPID ANTIBODY

Experimental studies

Localized E. coli infection in animals was found to cause a transient endotoxemia in the absence of bacteremia, and induced a protective antibody response to the endotoxin independent of antibody formation; 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. 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 dival O polysaccharide. The antisera obtained from immunization with this vaccine was able to prevent LPS-mediated toxicity, asmanifested by (1) a lowering of mortality rate in experimental animals following LPS intoxication, and (2) significant reduction in both local (dermal) and generalized (DIC) Shwartzman reactions initiated by LPS (reviewed in ). This antisera was also able to protect against lethal bacteremia caused by Klebsiella, Pseudomonas and E. coli in a neutropenic rabbit model of sepsis. 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 Chedd1 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.

In an extensive series of studies, McCabe and colleagues found that both active and passive immunization with an Rf 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. These studies by Chedd, 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. Of 3 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, ). Among the 18 patients whose hypotension required pressors for at least 6 h, 27 (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 antisera significantly decreased the incidence of shock and more significantly, death from shock (relative risk in controls was 2.3 and 4.2, respectively, 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.

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. This result should not have been anticipated since results of the initial clinical study and an experimental study 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 be 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 bacteria, 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 an 18 patient study. In a study of 55 patients, Schad et al. reported that administration of a monoclonal, non-hyperimmune immunoglobulin preparation that contained IgG, IgA, and IgM isotypes (Pentaglobin, Boehringer, 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.

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. 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. 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 a similar non-IVIG 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 polyclonal 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.

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.

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.

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. 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 MAb.

While many MAbs to core glycolipid structures have been described in the literature, two antibodies, E5 (Xoma, Berkeley, CA, USA) and HA-1A (Centocor, Malvern, PA, USA) have been investigated in both preclinical and clinical studies of sepsis. 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. 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.

Using fluid phase radio-immunoassay, Warren et al. have shown that both MAbs bind tightly to smooth LPS molecules of different serotypes only when the antibodies are present in high concentrations.
The abilities of these MAbs to bind to heterologous smooth LPS serotypes, however, do 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 has unmasked the core structure and allowed binding of the MAbs.\textsuperscript{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.\textsuperscript{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.\textsuperscript{52,53} These observations cast doubt on the therapeutic relevance of these MAbs in the treatment of septic shock.\textsuperscript{44,51,53}

**Activity in animal models**

MAb E5 improves the hemodynamics and physiologic parameters following endotoxin challenge in a sheep model.\textsuperscript{51} This MAb was also able to provide modest protection from lethality, particularly when accompanied by antimicrobial agents, in bacteremic models in mice and rats.\textsuperscript{44,47} Survival benefits from the use of HA-1A were reported in neutropenic rabbit and mouse peritonitis models.\textsuperscript{45,48} The HA-1A MAb also prevented the dermal Schwartzman reaction in rabbits.\textsuperscript{45} In contrast, Baumgartner et al.\textsuperscript{54} 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.\textsuperscript{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,\textsuperscript{56} then no animal model, including subhuman 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.\textsuperscript{57}

**Clinical trials**

The initial phase III clinical trials with both MAbs were reported in 1991 and have been extensively commented upon.\textsuperscript{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

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

<table>
<thead>
<tr>
<th></th>
<th>E5 (n = 468)</th>
<th>HA-1A (n = 543)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo</td>
<td>Placebo</td>
</tr>
<tr>
<td></td>
<td>(2 mg/kg x 2)</td>
<td>(100 mg x 1)</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>P value</td>
</tr>
<tr>
<td>Total</td>
<td>NR (40%)</td>
<td>262 (39%)</td>
</tr>
<tr>
<td></td>
<td>NR (41%)</td>
<td>281 (43%)</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Age (mean)</td>
<td>60.1</td>
<td>58.0</td>
</tr>
<tr>
<td></td>
<td>63.4</td>
<td>62.3</td>
</tr>
<tr>
<td></td>
<td>&lt; 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>APACHE II (mean)</td>
<td>16.9</td>
<td>23.6</td>
</tr>
<tr>
<td></td>
<td>17.3</td>
<td>25.7</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>% male</td>
<td>66%</td>
<td>59%</td>
</tr>
<tr>
<td></td>
<td>66%</td>
<td>58%</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>% in shock</td>
<td>55%</td>
<td>51%</td>
</tr>
<tr>
<td></td>
<td>59%</td>
<td>51%</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>% ARDS</td>
<td>20%</td>
<td>9%</td>
</tr>
<tr>
<td></td>
<td>23%</td>
<td>13%</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>% ARI</td>
<td>23%</td>
<td>35%</td>
</tr>
<tr>
<td></td>
<td>22%</td>
<td>40%</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>% DIC</td>
<td>29%</td>
<td>18%</td>
</tr>
<tr>
<td></td>
<td>25%</td>
<td>21%</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Patients with GNB (n)</td>
<td>94</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>77</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>% mortality in GNB</td>
<td>NR</td>
<td>30%</td>
</tr>
<tr>
<td></td>
<td>NR</td>
<td>49%</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.014</td>
</tr>
<tr>
<td>% mortality in GNB: shock</td>
<td>NR</td>
<td>33%</td>
</tr>
<tr>
<td></td>
<td>NR</td>
<td>57%</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.017</td>
</tr>
<tr>
<td>% mortality in GNB: no shock</td>
<td>NR</td>
<td>27%</td>
</tr>
<tr>
<td></td>
<td>NR</td>
<td>40%</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.28</td>
</tr>
<tr>
<td>% mortality in GNI</td>
<td>38%</td>
<td>NR</td>
</tr>
<tr>
<td></td>
<td>41%</td>
<td>NR</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>% mortality GNI: no shock</td>
<td>30%</td>
<td>43%</td>
</tr>
<tr>
<td></td>
<td>43%</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>% mortality GNI: shock</td>
<td>45%</td>
<td>40%</td>
</tr>
</tbody>
</table>

* 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.

DVT: disseminated intravascular coagulation.

GH: Gram negative bacteraemia.

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.\(^4\) as in the first \(^{15}\) study.\(^{2,5}\) The E5 MAAb, 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.\(^6\) 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 \(P = 0.0241\); or in patients who had Gram-negative sepsis and were not in shock who received E5 (54% E5 vs 30% placebo \(P = 0.03)\). 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 fall 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) \(P = NS\); however, resolution of organ dysfunction was significantly more likely to occur with E5 treatment, as was true in the first study.\(^6\)

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 rate 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 human.\(^{62,64}\) 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.\(^6\) Another MAb developed by Sandoz, SDZ219-800, is a chimeric human-murine MAb which is broadly cross-reactive against smooth and rough LPS.\(^6\) 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\(^6\) in addition to inhibiting the LPS priming of human neutrophils for superoxide production.\(^6\) A murine IgM MAb, clone 20, binds to the \(\alpha\)-linked KDO (2-keto-3-deoxyoctulosonic acid) moiety of the core glycolipid and appears to provide protection against endotoxin-induced lethality in mice;\(^6\) 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.\(^6\) Another MAb that recognized a KDO epitope, GL11, protected against heterologous LPS lethality in sensitized mice even when given after LPS challenge.\(^7\) MAbs have been described that not only inhibit LPS-induced cytokine secretion and lethal shock, but also B cell mitogenesis.\(^7\)

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. 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. 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 MAb 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.

**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 difficult 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. 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 antisera 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 protein biologic manifestations of LPS include the induction of non-antibody moieties capable of inactivating LPS, and (3) the inability of anti-core glycolipid antibodies to bind to endotoxin of smooth, bactereicidal strains.

**Lack of reproducibility of protective effect**

It is now clear that an anti-endotoxin antisera may function by at least 3 different mechanisms: direct neutralization of the biological activity of the LPS in a manner similar to polymixin B; promotion of the clearance of the LPS from the circulation, or mediation of the opsonophagocytic killing of the bacteria. While it is possible to assess the ability of an anti-endotoxin antisera 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

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**Table 2: Activity of other anti-core glycolipid monoclonal antibodies**

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Source</th>
<th>Isotype</th>
<th>Epitope</th>
<th>In vitro activity</th>
<th>In vivo activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone 20</td>
<td>Murine</td>
<td>IgM</td>
<td>KDO from Re</td>
<td>Binding to smooth LPS</td>
<td>Protects mice from E. coli challenge</td>
<td>Appelmekh</td>
</tr>
<tr>
<td>1D4.38</td>
<td>Murine</td>
<td>IgG</td>
<td>35 core glycolipid</td>
<td>Inhibits TNF production</td>
<td>Inhibits TNF; protects mice from E. coli</td>
<td>Vacheron</td>
</tr>
<tr>
<td>N 2/26 20</td>
<td>Murine</td>
<td>IgM</td>
<td>Lipid A</td>
<td>Inhibits LPS priming of neutrophils</td>
<td>Inhibits TNF; protects mice from LPS injection</td>
<td>Cornelissen</td>
</tr>
<tr>
<td>FSW</td>
<td>Human</td>
<td>IgM</td>
<td>Lipid A</td>
<td>Binds to LPS, promotes opsonization and promotion of bactericidal effect</td>
<td>-</td>
<td>Winkelhake</td>
</tr>
<tr>
<td>MIA 1</td>
<td>Murine</td>
<td>IgM</td>
<td>Lipid A from Re</td>
<td>Inhibits IL-1, TNF; B cell matogenesis</td>
<td>Protects mice from lipid A</td>
<td>Ramachandra</td>
</tr>
<tr>
<td>SDZ 219 800</td>
<td>Chimera human mouse</td>
<td>IgG1</td>
<td>Core glycolipid</td>
<td>Inhibits LPS production, TNF, IL-6 secretion</td>
<td>Blocks rabbit pyrogen, LPS lethality in mice</td>
<td>Di Padova</td>
</tr>
<tr>
<td>SD5 117 15</td>
<td>Human</td>
<td>IgM</td>
<td>Phosphate fatty acid of lipid A</td>
<td>Binding to smooth LPS</td>
<td>-</td>
<td>Kazemi</td>
</tr>
<tr>
<td>GFL 11</td>
<td>Murine</td>
<td>IgG2b</td>
<td>KDO from Re</td>
<td>Binds to Re LPS</td>
<td>Protects mice from LPS challenge</td>
<td>Nys</td>
</tr>
</tbody>
</table>

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the dermal Shwartzman reaction by J5 antisera was not correlated with enhanced clearance of endotoxin from the circulation (as was previously observed with the induction of endotoxin tolerance); however, the beneficial effect of J5 antisera during infection was associated with an accelerated clearance of bacteria from the circulation. 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, in many models the dose-response curves between 100% survival and 100% death are quite steep (often occurring over a 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 endotoxin; 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 endotoxemia, 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. Also, with the infusion of large inocula it is difficult to elute 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. 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. Survival in these studies was better correlated with the receipt of post-immunization serum than with the actual level of antibody. 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 antisera.

Both retrospective serological surveys and experimental studies 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 ≥ 1:80 at the onset of bacteremia. This was independent of any contribution of O-specific IgG antibody which was also associated with a reduction in complications of Gram-negative bacteremia. 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. 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 sera 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. 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. Even though absorption of the antiserum 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. 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.

In contrast, fractionation of lapine anti-J5 antiserum 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 antiserum 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. 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 Re or J5 LPS.

Administration of endotoxin induces acute phase reactants (e.g., LPS binding protein, lipoproteins, soluble CD14, cytokines and perhaps other monocytes that may alter LPS activity. Warren and colleagues found non-antibody monocytes of human plasma capable of neutralizing the Limulus reactivity of LPS as well, if not better, than anti-endotoxin antibody. The above studies 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 immunophylactic 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 to not broadly protective anti-glycolipid antibody but rather to the well known polyclonal antibody response following endotoxin administration; 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.

If subsequent data confirm that anti-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. Following natural exposure, the human antibody response to core glycolipid antigens is modest compared to O antibody responses 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 antibodies to other core epitopes. Experimental studies described above achieved highly significant protection in animal models with antibodies directed against both Re and J5 LPS.

Similarly, serological surveys of bacteremic patients correlated level of anti-J5 and anti-Re 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. 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. 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 Re through Rc (but not Rd and Re) chemotypes. 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.

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 amphiphatic 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. Thus, as bacteria grow in broth, rate nephelometry assays can detect binding of anti-endotoxin antibody (HA-IA) to dividing bacteria, and this is inhibited by preincubation of the MAb with lipid A. Thus, pretreatment of smooth bacteria with inhibitory concentrations of antibiotics has been shown to expose core LPS epitopes to antibody binding. 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, 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. 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 assure 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.

**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. Despite the effectiveness of all 3 regimens, subsequent studies were conducted with serum collected at the height of the hemagglutinating (HGA) 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. 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. 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. We found that anti-core glycolipid antibody harvested after booster immunization may have better activity than antibody harvested after a primary series. 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.

**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, anti-TNF MAb and IL-1ra. 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 antisepsis 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, 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 antisepsis 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 documented 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 modulation by the drug might not be appropriate for the purpose of determining whether the drug has efficacy and might mask a clinical effect of 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 physiologic 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 are 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 of capsular) may hasten bacterial clearance and minimize later septic complications. After this stage, endotoxin, liberated either by growing bacteria or by treatment with antibiotics, can 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 H-1ra, are designed to intervene at this, but not earlier stages. In fact, there is evidence that anti-cytokines may be detrimental if given in the early phases of infection. Anti-endotoxin antibodies will reverse this 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 H-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. 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.

Where 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 agents with which to treat or prevent sepsis.

References


7. Favaud GO, Morgan HR, Eff. Produced by intravenous injection in man of a toxic antigenic material derived from Pseudomonas aeruginosa.

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7. Favaud GO, Morgan HR, Eff. Produced by intravenous injection in man of a toxic antigenic material derived from Pseudomonas aeruginosa.
Treatment of septic with antibody to endotoxin

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