Old Herborn University Seminar Monograph 17:
Possibilities for active and passive vaccination against opportunistic infections.
Editors: Peter J. Heidt, Richard I. Walker, Volker Rusch, and Dirk van der Waaij.
Herborn Litterae, Herborn-Dill, Germany: 19-30 (2004).

DEVELOPMENT OF AN ANTI-CORE LIPOPOLYSACCHARIDE VACCINE FOR THE PREVENTION AND TREATMENT OF SEPSIS*

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SUMMARY

Sepsis continues to be a leading cause of death among hospitalised patients. Despite advances in supportive care and the availability of potent antimicrobials, the mortality exceeds 20%. The passive infusion of antibodies directed against a conserved region of the lipopolysaccharide (LPS) of Gram-negative bacteria was highly protective in an early study (*Ziegler* et al., 1982). When this and similar preparations were unable to show consistent efficacy, efforts were directed towards other strategies, including cytokine modulation. Our group found that a whole bacterial vaccine made from the *E. coli* O111:B4, J5 (Rc chemotype) mutant induced protective antibodies when given passively as treatment for sepsis in a neutropenic rat model. A subunit vaccine, composed of detoxified J5 LPS complexed to group B meningococcal outer membrane protein (OMP), provided similar protection when antibodies were given passively, or induced actively in both the neutropenic and caecal ligation/puncture models of sepsis. A phase I study in 24 subjects (at 5, 10 and 25 μg doses [based on LPS] for each group of 8) revealed the vaccine to be well-tolerated with no systemic endotoxin-like effects. Although a 2-3 fold increase in antibody levels over baseline (by ELISA assay) was observed at the 10 and 25 μg doses, the plasma from both high and low responders reduced LPS-induced cytokine generation in whole blood. Re-immunisation of 6 subjects at 12 months did not convert low responders to high responders or boost the still elevated anti-J5 LPS levels of high responders. If functional assays of anti-LPS antibodies are better predictors of vaccine efficacy than ELISA antibody levels, then it will be necessary to determine which of many potential assays best correlates with protection in animal models. We are currently comparing a panel of functional assays with protective efficacy in animal models of sepsis, as well as the ability of adjuvants to enhance vaccine efficacy. The availability of an effective anti-endotoxin vaccine will provide additional therapeutic options for the prevention and/or treatment of sepsis.

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INTRODUCTION

Sepsis, a leading cause of death in intensive care units, has increased in frequency over the last two decades (*Martin* et al., 2003). Between 1979 and 2000 there was a four-fold increase in the number of cases of sepsis (from 164,000 to nearly 660,000). The mortality remains nearly 20% despite advances in supportive care and the introduction of potent antimicrobial agents (*Martin* et al., 2003). Consequently, additional therapeutic measures have been sought. The important role of Gram-negative bacterial lipopolysaccharide (LPS) in the pathogeneses of sepsis was recognised in the 1960's and 70's (*Braude* et al., 1960); therefore, it is not surprising that initial attention to adjunctive treatment measures focused on this molecule. Elucidation of the structure of LPS revealed that the lipid-A portion was highly conserved among species of *Enterobacteriacea*e and that the core regions also had considerable conservation. As a result, it was hypothesised that antibodies against these conserved LPS structures might provide protection against a broad range of Gram-negative bacteria. Investigators developed bacterial strains in which the core region of LPS was available to the immune system (i.e. not shielded by O antigen, for example *S. minnesota* Re595 [Re chemotype] and *E. coli* O111:B4, J5 mutant [Rc chemotype]) (*McCabe*, 1972; *Ziegler* et al., 1973). Pre-clinical work with anti-core LPS antibodies induced by these killed bacterial strains was effective in animal models of sepsis (*Ziegler* et al., 1975; *Johns* et al., 1983). In this manuscript we shall briefly review earlier studies with anti-endotoxin antibodies, and then describe our own studies with a detoxified J5 LPS (dLPS)/group B meningococcal outer membrane complex (OMP) vaccine that progressed to a phase I study in human subjects.

Early studies with anti-endotoxin antibodies

Based on these studies, *Braude* and colleagues prepared a whole bacterial vaccine by boiling *E. coli* 0111: B4, Rc chemotype (hereafter, J5 mutant) and raised immune sera in healthy volunteers. In a multi-centre clinical trial, patients with suspected Gram-negative bacterial sepsis were given either pre or post-immune sera in addition to standard therapy (*Ziegler* et al., 1982). Patients with Gram-negative bacteraemia who received post-immune sera had a better survival rate (22/91 [24%]) than those receiving pre-immune sera (30/100 [38%] p=0.041). Among those with either hypotension or in profound shock, there were even more significant differences in favour of the post-immune sera. Despite the therapeutic benefit, there was no evidence that the antisera prevented infection. In this trial investigators were unable to determine whether the antibody fraction of sera was responsible for the improved survival. Further, the antigen in the whole bacterial vaccine responsible for inducing the protection was not clearly identified. Finally, since the "therapeutic product" was material from an individual volunteer and not a reproducibly made reagent, this clinical study must be viewed as a proof of principle rather than the testing of a potential therapeutic product.

Subsequent investigators were unable to confirm the findings of *Ziegler* and colleagues; however, none of these studies were similar in design to the original study and none clearly documented the maintenance of anti-endotoxin antibodies (Table 1). In one study

Table 1: Passive administration of anti-core LPS antibodies for sepsis: Previous clinical studies

Study	Product	Number of patients	Ab levels	Outcome
Ziegler (1982)	J5 serum	91	Increased	Reduced mortality, especially if shock
Baumgartner (1985)	J5 Plasma	126	Not done	9/136 controls vs. 2/126 patients died
J5 Study Group (1992)	J5 plasma	40	No increase	No protection in meningococcemia
Commetta (1992)	Screened IVIG	108	Consumption	No protection
Calandra (1988)	J5 IVIG	30	Not done	No effect
Schedel (1991)	"Enriched" IVIG	27	Consumption	Titre-related protection 1/27 vs. 9/28 survival
Fomsgaard (1989)	Screened IVIG	9	Consumption	Anti-LPS IgG reduced TNF

children with meningococcal purpura fulminans were given J5 plasma at the onset of illness (*J5 Group*, 1992). There was no evidence of benefit; however, there was no increase in anti-J5 LPS antibody when measured at 6 hours after infusion. In another study, use of J5 plasma was ineffective when given as prophylaxis to surgical patients. This study confirmed the earlier finding of *Ziegler* et al. (1982) that J5 serum did not prevent the development of Gram-negative infection (*Baumgartner* et al., 1985). Similarly, in another clinical trial IgG was prepared from the plasma of volunteers who were immunised with the whole bacterial J5 vaccine (*Calandra* et al., 1988). A single infusion of IVIG was ineffective in a clinical trial of patients with sepsis; however, there appeared to be only a two-fold response in anti-J5 LPS antibody in the starting material before fractionation into IVIG. Thus, although the level of anti-core LPS antibodies after infusion was not measured in these patients, it is unlikely that adequate levels of anti-J5 IgG were administered. In yet another study, plasma from blood donors was screened for high levels of naturally occurring anti-core LPS (*S. minnesota,* Re 595*)* antibody and high titred material was pooled and made into an IVIG (*The Intravenous Immunoglobulin Collaborative Study Group*, 1992). This preparation was compared to standard IVIG in its ability to prevent the onset of sepsis when given as prophylaxis to patients who underwent surgery. In the absence of documented infection, the levels of antibody at 2 days was <50% that of levels obtained at 2 hr post infusion (*The Intravenous Immunoglobulin Collaborative Study Group*, 1992). This enriched anti-core LPS IVIG was unable to prevent infection, sepsis or death. Thus, in all of these studies it is likely that inadequate amounts of antibodies were given or inadequate levels of antibody were maintained to test the hypothesis that anti-endotoxin antibodies were effective in the treatment of sepsis.

A number of studies (*Pollack* et al., 1983; *Goldie* et al., 1995; *Zinner* and *McCabe*, 1976) have clearly established a relationship between the level of anti-core LPS antibody at the onset of sepsis and outcome. More importantly, a decrease in anti-core LPS antibody during a septic episode forebodes a poor out-

come (*Fomsgaard* et al., 1989; *Schedel* et al., 1991; *Nys* et al., 1993; *Goldie* et al., 1995). Consequently, in the absence of documentation that there was an adequate level of circulating anti-endotoxin antibodies, it is difficult to exclude the hypothesis that anti-endotoxin antibodies might be an effective adjunctive therapy for sepsis. Indeed, in small studies, both *Schedel* et al. (1991) and *Fomsgaard* et al. (1989) each demonstrated that maintenance of "adequate levels" of anti-CGL antibody with multiple infusions corresponded to a decrease in circulating endotoxin levels and increased survival.

Despite the fact that early studies with antisera to lipid-A were unsuccessful in treating sepsis in animal models (*Bruins* et al., 1977), nevertheless, monoclonal antibodies to lipid-A were developed and tested in clinical trials without success (*Greenman* et al., 1991; *Ziegler* et al., 1991). Given the repeated failures of anti-core LPS and anti-lipid-A antibodies to affect the outcome of sepsis in clinical trials, subsequent efforts were directed towards the rapidly developing field of cytokine modulation.

Additional therapeutic strategies

Recognition of the important role of TNF-α and IL-1 in the development sepsis resulted in multiple clinical trials in which inhibitors of TNF and IL-1 activity were tested for therapeutic efficacy in sepsis. After many trials with these and other endogenous mediators of sepsis, no convincing therapeutic effect was detected (*Zeni* et al., 1997). In contrast to studies with anti-endotoxin antibodies that target an invading pathogen, however, administration of active cytokine antagonist often was associated with increases in lethal infections. These unforeseen adverse events illustrate the difficulty in trying to "fine-tune" the levels of endogenous mediators of sepsis in the host as opposed to efforts to target microbial initiators of sepsis. In view of the difficulties in trying to monitor the effect of therapy on host-defences as well as the success of the initial clinical trial with J5 antiserum, we decided to re-examine the potential utility of anti-core endotoxin antibodies, such as the J5 antibody. This effort was facilitated by the development of a neutropenic rat model of sepsis in which animals developed a lethal bacterial infection following the administration of relatively low doses of opportunistic pathogens (*Collins* et al., 1989).

CURRENT STUDIES WITH ANTI-J5 ANTIBODY

We obtained the *E. coli* 0111:J5 strain from Dr. Ziegler and prepared a heat-killed whole bacterial vaccine according to the original method. Antisera raised in rabbits with this vaccine were highly protective in a neutropenic rat model of sepsis, when given at the onset of fever (*Bhattacharjee* et al., 1994) (i.e. as therapy). The effect was clearly dose-related (*Bhattacharjee* et al., 1994), which lent credence to the argument that previous clinical trials with anti-endotoxin antibodies may not have been successful because of inadequate levels of serum administered. We further showed that IgG was the protective fraction in serum and was directed against the core J5 LPS in the whole bacterial vaccine (*Bhattacharjee* et al., 1994). Six of 8 animals that received affinity purified J5 LPS-specific IgG were protected against lethal *Pseudomonas* sepsis vs. none of 25 animals receiving pre-immune IgG. Importantly, the protection was clearly dose-related with animals receiving 9 ml/kg IgG protected versus none re-

Table 2: Local and systemic reactions following immunisation with dJ5 LPS/OMP vaccine

Reactions	Dose (based on dLPS)		
	5 μg	10 μg	25 μg
Local			
Erythema	2[a]	1	3
Induration	2	0	4
Swelling	2	8	6
Pain			
Severe	0	0	0
Moderate	8/1[b]	7/0	12/0
Mild	10/5	12/5	9/8
None	6/18	5/19	3/16
Analgesia	2	2	2
Systemic			
Fever	1	1	1
Headache	2	1	0
Fatigue	0	0	0
Haematologic			
Anaemia	0	1	0
Leukopenia	0	0	0

Volunteers were immunised at day 0, day 28 and day 56 with the indicated dose.
[a]number of reactions per 24 total immunisations (8 subjects, 3 doses).
[b]number of reactions at day 1/day2 after immunisation.

ceiving <6 ml/kg (*Bhattacharjee* et al., 1994).

Based on these findings we made a J5 LPS vaccine, which was detoxified by removing the ester-linked fatty acids through alkaline treatment (*Bhattacharjee* et al., 1996). The LPS was not immunogenic when given alone, with alum, with QS21 or when conjugated to tetanus toxoid. When complexed non-covalently with the outer membrane protein of group B-meningococcus, however, the formulation was highly immunogenic in mice, rabbits and rats. Antisera raised with this vaccine was highly protective in a neutropenic rat model after challenge with either *Klebsiella* or *Pseudomonas* when the antibody was given either as passive therapy at the time of sepsis, or when antibodies were actively induced by immunisation before the start of sepsis. In the latter instance, immunisation with this vaccine did not prevent bacteraemia, but did reduce mortality. Receipt of anti-J5 antibody reduced circulating levels of endotoxin at 24 hr after infusion and reduced the circulating TNF levels compared to the effect with pre-immune sera (*Bhattacharjee* et al., 1996). Active immunisation with the J5dLPS/OMP vaccine promoted the uptake of bacteria from the circulation and killing (i.e. decreased organ bacterial load). Immunisation both actively and passively was also protective in another animal model of sepsis, caecal ligation/puncture in mice. This model differs from the neutropenic rat model in that the sepsis is polymicrobial. With these findings we prepared a vaccine for human use.

Phase I clinical study

A Phase I study (*Cross* et al., 2003) was conducted in 24 healthy subjects. Subjects received either 5, 10, or 25 μg

Table 3: Anti-J5 LPS ELISA titres of sera from volunteers in the phase I trial

	IgG			IgA		
Group[a]	Pre	Post	Fold rise	Pre	Post	Fold rise
5μg	1.7[b] ± 0.28	3.6 ± 0.71	2.0 ± 0.18	1.3 ± 0.14	2.6 ± 0.3	2.1 ± 0.3
10μg	2.8 ± 0.5	5.8 ± 1.9	3.3 ± 0.4	4.4 ± 0.6	9.1 ± 2.0	2.0 ± 0.3
25μg	2.1 ± 0.18	4.9 ± 0.6	2.3 ± 0.3	1.8 ± 0.3	3.9 ± 0.9	2.2 ± 0.5

	IgM		
Group	Pre	Post	Fold rise
5μg	11.2 ± 0.9	16.9 ± 1.3	1.5 ± 0.1
10μg	18.9 ± 4.8	66.2 ± 24.0	3.2 ± 1.0
25μg	6.5 ± 1.1	18.2 ± 5.4	2.9 ± 0.6

[a] 8 volunteers in each group received J5 dLPS/OMP vaccine at time 0, days 28 and 56.

[b] Serum antibody levels were measured according to our previously described methods (*Cross* et al., 2003). Data represent mean ± SEM optical density units (ODU). ODU are defined as the product of the optical density and reciprocal titre for the serum dilution that gives an optical density closest to but still below 1.00 (e.g. OD 0.400 at 1:100 dilution = 40 ODU). Post levels are from the peak antibody level measured on specimens obtained up to 3 months after immunisation. Fold-rises were calculated for each subject and a geometric mean-fold rise for each group then determined.

of vaccine (based on LPS content) at time 0, 1 and 2-months (i.e. 3 total doses). There were few systemic responses (headaches/fever/fatigue) (Table 2). No temperatures >99.9°F were recorded. Most individuals had a mild-to-moderate degree of tenderness at the injection site, which usually resolved by 48-hours. For comparison, the only study to report the incidence of adverse effects with the heat-killed J5 vaccine observed 7/16 incidences of systemic reactions to the initial vaccine, and 3/9 subjects who returned for a second dose (*Schwartzer* et al., 1988). No abnormalities were seen in renal (creatinine, urinalysis), liver (serum alkaline phosphatase, transaminases, bilirubin) or haematologic (leukopenia, anaemia) studies compared to baseline studies (data not shown).

Antibody responses were measured by ELISA (Table 3). Compared to pre-immunisation levels there was a mean 3-fold increase in IgG and IgM levels in the 10 μg group. The 5 μg and 25 μg dosage groups had slightly lower responses. Subjects in all groups had higher baseline levels of IgM antibody to core LPS. We did not assess the affinity of the pre- vs. post-immune anti-core LPS antibodies. Six subjects (3 high and 3 low responders) received a single booster dose of 25μg of vaccine at 12-months to see if it were possible to convert non-responders and to boost the level of responders. High responders were defined as having >2.5 fold increase in serum IgG over baseline, while low responders had <2 fold increase. At 12 months, among responders, pre-boost levels of antibody were still elevated but had decreased by approximately 50%. There was no increase in antibody levels among the high responders following the booster dose. Subjects who did not respond after the primary series did not convert with the

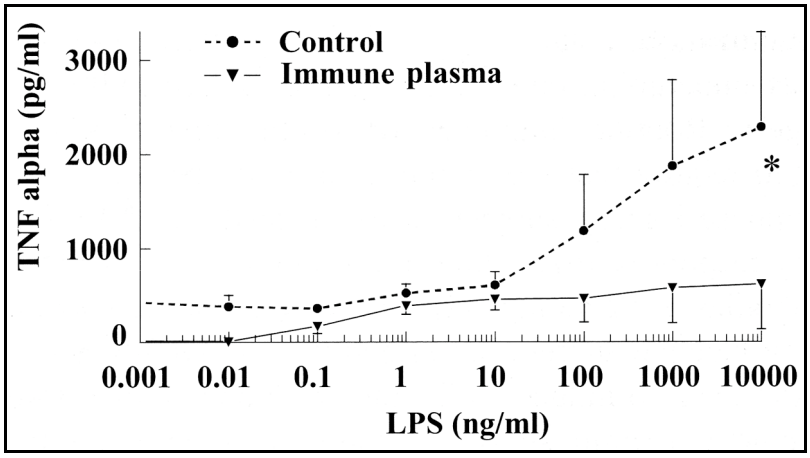

Figure 1: Effect of pre-incubation of LPS with either post-immunisation plasma or control pooled plasma from non-immunised individuals. Different doses of *E. coli* LPS were added to plasma from either one subject with >3-fold increase in anti-J5 dLPS antibody levels or to control plasma. Then the mixture was added to heparinised whole blood from a J5 LPS-naïve donor. The blood was incubated for 24 hr and the supernatant analysed for TNFα.

booster dose. Plasma from all six subjects was obtained one week after the booster dose. These were evaluated in functional assays.

Functional studies of anti-J5 LPS antibody

With most other vaccines there is usually one functional assay recognised as corresponding to vaccine efficacy. For example, opsonic antibody assays for pneumococcal immunisation are thought to better reflect vaccine efficacy than ELISA (*Johnson* et al., 1999; *Kim* and *Seoh*, 1999). Viral neutralisation assays or serum bactericidal tests have also been thought to correspond to efficacy for other vaccines. In the case of an anti-endotoxin vaccine, however, it is not readily apparent what functional assay would best reflect vaccine efficacy. Many functional activities are initiated by LPS, including induction of cytokines, fever, and coagulation as well as the initiation of complement cascades, among a great many other activities. We tested the plasma of the six volunteers in the Phase I study (three high and 3 low responders) who received a booster dose (25 mg) of vaccine at one year in an *ex vivo* cytokine assay. In this assay, LPS is added to heparinised whole blood and incubated at 37°C for 24-hours (*Kovach* et al., 1990). Cytokine generation was then measured in the supernatant. When LPS was pre-mixed with post-immune plasma before addition to the blood, there was a highly significant decrease in TNF (Figure 1) and in IL-6 generation (data not shown) compared to LPS that was exposed to control plasma (*Cross* et al., 2003). This was observed for both low and high responders. When plasma was diluted, however, the higher titred plasma had more activity. Consequently, although the ELISA antibody level did not correlate with functional activity, those with higher antibody levels did appear to have a higher LPS neutralising capacity. In a preliminary study, the plasma from a high responder enhanced the clearance of bacteria and endotoxin from the circulation of rats (*Cross* et al., 2003).

In yet another functional assay of LPS activity, pre-incubation of human

Table 4: Post-immune rabbit sera block LPS-primed superoxide response of human neutrophils

Pre-treatment	Change in OD_{560}	%Control	Anti-J5 LPS IgG (ng/ml)
None	0.023 ± 0.001		not applicable
LPS/HBSS	0.199 ± 0.033		not applicable
LPS/NRS	0.212 ± 0.005	100	109
LPS/anti-J5-1	0.143 ± 0.042	67	727
LPS/anti-J5-2	0.161 ± 0.012	76	1528
LPS/anti-J5-3	0.144 ± 0.020	68	473

Human PMNs were suspended in HBSS/2% human serum and incubated for 60 min at 37°C in medium, medium and LPS or rabbit serum with LPS. The serum from 3 different rabbits (anti-J5-1; anti-J5-2 and anti-J5-3) immunised, or from non-immunised rabbits (NRS) were used (Anti-J5 LPS antibody levels for each rabbit are indicated in the last column). After washing, the PMNs were stimulated with FMLP (10^{-7} M) for 10 min in the presence and absence of superoxide dismutase and the change in ferricytochrome C reduction between 0 and 10 min samples determined by absorption at 550 nm. NRS=normal rabbit serum; HBSS=Hank's Balanced Salt Solution. Each condition performed in triplicate. Representative experiment shown of 3 with similar results.

neutrophils with LPS primes the ability to generate superoxide in response to a neutrophil agonist, formyl-methionyl-leucyl-phenylalanine (fMLP) (*Guthrie* et al., 1984). Pre-incubation of LPS with post-immune sera from three different rabbits (anti-J5-1through 3) immunised with the J5dLPS/OMP vaccine reduced the ability of LPS to prime this response (Table 4). Although there did not appear to be an antibody dose-related inhibition of LPS priming based on ELISA antibody levels, we did not dilute out the antisera. When this was done in the *ex vivo* cytokine induction assay, differences were observed (*Cross* et al., 2003). Based on these initial studies we plan to compare the ability of high and low responder plasma to protect in the caecal ligation puncture and neutropenic rat models of sepsis, to recognise heterologous LPS in other binding assays (fluid phase, and binding to whole bacteria by flow cytometry) and to neutralise the ability of LPS to induce cytokines by THP1 and RAW cells *in vitro*. These studies may provide data as to which functional assay may correlate best with protection in animal model of sepsis. This becomes an even more important consideration since there has been considerable and ongoing debate on the methodology for measuring anti-LPS antibodies by ELISA (*Warren* et al., 1993).

PROPOSED USE OF ANTI-ENDOTOXIN VACCINE

If an effective anti-endotoxin vaccine were available for the prevention and/or treatment of sepsis, then it might be used in several different conditions. Several populations are at higher risk of sepsis and might be considered for immunisation: soldiers, police, fire fighters, as well as patients undergoing complicated abdominal or genitourinary surgery. Routine immunisation of the first three groups would require that the antibody response be long-lived. In our phase I study, subjects with elevated anti-J5 LPS antibody responses after initial immunisation still had elevated antibodies at 12 months (*Cross* et al.,

2003). In the case of patients undergoing elective surgery, an effective anti-endotoxin vaccine would need to induce antibodies after one or two doses of vaccine. Co-administration of the vaccine with an adjuvant might accelerate the antibody response in a manner similar to that of the oligonucleotide, CpG, given with hepatitis B (a vaccine also given in 3 doses) (*Davis* et al., 2000). Since after acute injury there is a Th2 polarisation, patients admitted with burns or trauma might respond to active immunisation (*Lyons* et al., 1997; *Ginnoudis* et al., 1998). We administered experimental *Klebsiella* and *Pseudomonas* vaccines to patients admitted following severe trauma and found that they responded well to both vaccines (*Campbell* et al., 1996).

Alternatively, anti-core LPS antibodies could be given passively to septic patients. In this instance, it would be essential to monitor the circulating levels of anti-core LPS antibodies. In our own pre-clinical studies in neutropenic rats there was a clear dose-related protection (*Bhattacharjee* et al., 1994), and previous clinical trials did not pay adequate attention to the maintenance of antibody levels. Additional doses of antibody may be required during a septic episode. In patients who become septic despite active immunisation with an anti-endotoxin vaccine, supplementation with passive administration of antibodies may be required to counter any consumption of antibody, as was documented in previous trials.

CONCLUSIONS

Our own bias is that many of these previous studies that investigated the efficacy of anti-endotoxin antibody therapy did not adequately measure the amount of antibody administered and did not insure adequate levels of antibody after initial infusions. Consequently, the potential role of anti-core endotoxin antibody therapy has not been sufficiently tested to discard the hypotheses. In monitoring the adequacy of therapy, the discrepancy between the ELISA antibody levels in human subjects and their activity in functional studies needs to be confirmed in a more rigorous fashion. Given the number of functional assays with which one might measure anti-endotoxin activity, this may become a daunting task. The conflicting data with previous studies of anti-endotoxin antibody therapy demands, however, that this effort be pursued in order to better evaluate the response to vaccine such as the one under present study. The current studies suggest that monitoring responses with functionally relevant assays may be an important component of clinical trials with anti-endotoxin antibodies. Moreover, our earlier studies in a neutropenic rat model of sepsis demonstrated the importance of giving adequate levels of anti-endotoxin antibodies (*Bhattacharjee* et al., 1994). The more recent study in human subjects found that even though the plasma from both high and low responders neutralised the cytokine-inducing activity of LPS, nevertheless, the activity was greater for the high responders (*Cross* et al., 2003). Consequently, it may be desirable to devise strategies to improve the antibody response with this J5 dLPS/OMP complex vaccine.

Future studies will be directed toward administration of this vaccine with adjuvants that may boost the level of anti-endotoxin antibodies and enhance the functional activity of the preparation. These strategies are currently being investigated.

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