Antibodies to Lipopolysaccharides after Immunization of Humans with the Rough Mutant *Escherichia coli* J5

J. D. Baumgartner, D. Heumann, T. Calandra, and M. P. Glauser

Division of Infectious Diseases, Department of Internal Medicine, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland

To investigate whether immunization with *Escherichia coli* J5 boiled cells induces antibodies directed at deep core structures, antibodies against J5 lipopolysaccharide (LPS), Re LPS, and lipid A were measured in the serum of 70 volunteers before and 2 weeks after immunization. To improve the sensitivity and the specificity of ELISA, complexes of core LPS with high-density lipoproteins were used instead of free core LPS as antigens. A median three-fold increase in antibodies directed against J5 LPS was observed, but no significant increase in the antibodies against Re LPS or lipid A was found. Since J5 antiserum did not react with several smooth LPS or with Re LPS and lipid A, cross-reactivity could not be demonstrated. Thus, immunization of volunteers with *E. coli* J5 produced a modest specific antibody response against J5 LPS. The mechanism of protection previously observed with J5 antiserum remains unclear.

Two clinical trials have suggested that human serum from volunteers immunized with the rough mutant *Escherichia coli* J5 (J5 antiserum) is effective as an adjunct in the treatment [1] or prevention [2] of gram-negative shock. However, the mechanism of protection against gram-negative septic shock afforded by J5 antiserum remains unknown. Rough mutants of gram-negative bacilli expose on their surface various parts of the core region of the lipopolysaccharide (LPS) that are hidden on smooth bacteria by the O-side chains and are therefore poorly accessible to immunologic reactions. Since the core LPS is relatively conserved phylogenetically, a working hypothesis is that anti-core LPS antibodies may cross-react with various gram-negative bacteria and possibly be protective against a wide range of gram-negative bacteria. However, the outcome of patients correlated weakly [1] or not at all (unpublished data) with the levels of J5 LPS antibodies measured in the sera administered.

J5 LPS is a complex molecule composed of core saccharides (heptose, glucose, *N*-acetylglucosamine, and 3-deoxy-D-manno-2-octulosonate [KDO]), of phosphate, ethanolamine, and of the toxic lipid A [3]. Immunization with J5 vaccine might therefore induce a mixture of antibodies against various epitopes of J5 LPS. A hypothesis raised to explain the lack of correlation between protection and levels of J5 LPS antibodies was that only antibodies directed to the immunodominant core sugars are detected when measuring J5 LPS

antibodies with an ELISA or with an indirect hemagglutination test. If the postulated cross-protective antibodies are directed at deeper core structures, such as lipid A or the lipid A–KDO region, they may remain undetected when the whole J5 LPS molecule is used as antigen in serologic tests, because these deeper structures may be hidden by the outermost core sugars.

To investigate this hypothesis, we measured by ELISA the antibody response to lipid A and Re LPS (composed of lipid A and KDO) after 70 healthy volunteers were vaccinated with *Escherichia coli* J5. Core LPS, especially Re LPS and lipid A, are hydrophobic structures with limited solubility and self-aggregating properties. They are difficult to use as antigens in ELISA because the coating of the plates may be poor and because nonspecific sticking of immunoglobulins to hydrophobic structures may be observed [4]. We have noted that complexing LPS to high-density lipoproteins (HDL), a natural carrier of LPS, modified the physicochemical state of core LPS and that use of HDL-LPS complexes to coat ELISA plates increased both the sensitivity and the specificity of the test compared with coating with free-core LPS [4].

In the present study, we used complexes of LPS with HDL instead of free LPS as coating antigens [4]. To study whether J5 vaccine produces a polyclonal antibody increase, we also measured the levels of antibody directed against LPS from *E. coli* O111 (O111 LPS) and against a mixture of LPS from seven different, smooth gram-negative strains and total IgG and IgM levels.

Materials and Methods

Immunization of volunteers. The procedure was performed as previously described [1]. *E. coli* J5 vaccine (provided by E. J. Ziegler, San Diego) was prepared from stationary-phase *E. coli* J5 bacterial cells grown in trypticase soy broth, harvested by centrifugation, washed three times in sterile 0.15 *M* sodium chloride, boiled for

2.5 h, adjusted to 5 × 10⁹ bacteria/ml, and conserved with 5 g/l
of phenol. Healthy volunteers were immunized subcutaneously with
two 1-ml injections of *E. coli* J5 vaccine simultaneously in two sepa-
rate sites and with two more 1-ml injections 48 h later. Plasma sam-
ples were collected before and 2 weeks after the first injections. The
plasma were used during a study of prevention of gram-negative septic
shock in high-risk surgical patients [2], and aliquots were kept at
−70°C for antibody determinations.

LPS. LPS from *E. coli* O111:B4 (O111 LPS,) *E. coli* O26, *E.
coli* O127, *Salmonella minnesota* S128, *Salmonella enteritidis,
Salmonella typhimurium,* and *Serratia marcescens* were obtained
from Sigma Chemical (St. Louis). LPS from *E. coli* J5 mutant (J5
LPS), LPS from *S. minnesota* R595 mutant (R595 LPS), and lipid
A from *S. minnesota* R595 mutant (R595 lipid A) were obtained
from Ribi Immunochem Research (Hamilton, MT). LPS from *E.
coli* D31m4 (D31m4 LPS) (an Re-type rough mutant) and the lipid
A extracted from it (D31m4 lipid A) were obtained from List Bio-
logical Laboratories (Campbell, CA). Lyophilized LPS were recon-
stituted in pyrogen-free water to a concentration of 1 mg/ml.
Triethylamine (0.5% vol/vol) was added for Re LPS and lipid A.

Determination of antibody levels by ELISA. Complexes of LPS
with HDL were used instead of free LPS as coating antigens (see
[4]). Controls with HDL alone were included and background values
from these wells were subtracted from the readings. Hyperimmune
rabbit antisera specific for *E. coli* O111, J5, D31m4 LPS, *S. min-
nesota* R595 LPS, and for lipid A extracted both from *S. minnesota*
R595 and from *E. coli* D31m4 were used to demonstrate the ade-
quate presence of antigens on the plates. The assay was standardized
with immunopurified human anti-J5 LPS IgG or IgM antibodies pre-
pared as described [4]. A standard curve was established with the
immunopurified antibodies for the determination of the levels of an-
tibodies in the test samples. For the purpose of this study, the same
standard curve was used to estimate the concentrations of all anti-
LPS antibodies measured simultaneously with J5 LPS antibodies.
The sensitivity of the assay was 0.5 μg/ml for IgG or IgM antibod-
ies. For antibodies to *E. coli* D31m4 LPS and lipid A, which were
assayed later, the results were reported as arbitrary units (AU; opti-
cal densities × dilutions).

IgG and IgM serum levels. Total IgG and IgM serum levels were
measured by standard methods using a Behring nephelometer ana-
lyzer (Hoechst-Behring).

Statistical methods. Antibody levels before and after immuni-
zation were compared by the nonparametric Wilcoxon paired-sample
test.

Results

Antibodies to J5 LPS. Of the 70 volunteers before immu-
nization, 60% and 96% had detectable levels of IgG and IgM
antibodies, respectively, against J5 LPS. These values increased
to 86% and 99% after vaccination with *E. coli* J5. The me-
dian increase in the levels of antibodies directed to J5 LPS
was 3-fold for IgG and a 3.25-fold for IgM (table 1). This slight
increase contrasted with the several hundredfold increase ob-
served in rabbits immunized intravenously: titers usually
increased from 1:100–1:200 before immunization to 1:25,600–
1:102,400 after immunization with boiled bacterial cells.

Table 1. Antibody levels in 70 volunteers immunized with
Escherichia coli J5.

| Antibody, antigen* | Median levels of anti-LPS antibody (μg/ml) | | | | |
	Before immunization (range)		After immunization (range)		Fold increase	P
IgG						
J5 LPS	4	(<0.5–87)	13	(<0.5–251)	3.25	<10⁻⁶
R595 LPS	5	(<0.5–116)	5	(<0.5–135)	1	NS
R595 lipid A	3	(<0.5–41)	3	(<0.5–69)	1	NS
O111 LPS	<0.5	(<0.5–30)	<0.5	(<0.5–50)	1	NS
Smooth LPS†	21	(<0.5–140)	26	(<0.5–117)	1.24	NS
IgM						
J5 LPS	3	(<0.5–33)	9	(<0.5–53)	3	<10⁻⁶
R595 LPS	1	(<0.5–8)	1	(<0.5–5)	1	NS
R595 lipid A	<0.5	(<0.5–3)	<0.5	(<0.5–2)	1	NS
O111 LPS	1	(<0.5–14)	1	(<0.5–14)	1	NS
Smooth LPS†	4	(<0.5–26)	4	(<0.5–27)	1	NS

NOTE. LPS, lipopolysaccharide.
* Complexes of high-density lipoproteins with LPS or lipid A were used to coat
plates.
† Mixture of LPS from seven smooth bacterial strains (see text).

Antibodies to Re LPS and to lipid A. Of volunteers before
immunization, 67% and 69%, respectively, had detectable
IgG and IgM antibodies against *S. minnesota* Re LPS and 59%
and 16% against *S. minnesota* lipid A. Overall, these values
did not increase significantly after vaccination, and there was
no significant increase of the antibody levels in the subgroup
of volunteers with detectable preimmune levels (table 1).

Since antigenic and structural differences exist between the
core regions of *S. minnesota* and *E. coli*, we measured anti-
bodies against the LPS of a Re mutant of *E. coli* (strain D31m4)
and against its lipid A in the sera of 21 volunteers randomly
selected from our series. Before immunization with *E. coli*
J5, the median (range) levels were 6 AU (0–64) for IgG and
2 (0–21) for IgM anti-D31m4 LPS antibodies, and 2 (0–31)
and 9 (0–55) AU, respectively, for anti-D31m4 lipid A anti-
bodies. There was no statistically significant increase in any
of these antibodies after immunization. In only four volun-
teers was some increase (less than threefold) in the levels of
one or several antibodies observed.

*Antibodies to LPS from smooth bacteria and total immu-
noglobulin levels.* No significant increase in specific IgG or
IgM antibodies against LPS from *E. coli* O111 (the parent
of the J5 mutant) was observed. A slight increase of the me-
dian IgG antibody level against the mixture of LPS from seven
smooth bacterial strains (1.24-fold) was not statistically
significant and of doubtful biological importance. IgM anti-
bodies directed against the same seven antigens did not in-
crease (table 1).

The total serum IgG or IgM levels were measured in 10
volunteers randomly selected from our group: The mean (SD)
IgG serum levels before and after immunization were 9.4 (1.4)

g/l and 9.2 (1.1) g/l, and the mean (SD) IgM serum levels were 1.0 (.04) g/l and 1.0 (.05) g/l, respectively.

Discussion

The current knowledge of the protective activity of antibody to endotoxin core was recently reviewed [5]. There is no direct and convincing proof so far that the protection observed with polyclonal antisera to rough mutants in animal experiments [6–8] is due to cross-reactive anti-LPS antibodies (see [9]). The protection reported with some monoclonal antibodies [10–12] needs confirmation [13–15]. In humans, there has been a discrepancy in clinical trials between the protection afforded by J5 antiserum and the weak or lack of correlation with the levels of J5 LPS antibodies measured in sera administered to patients.

In the present study, we found that immunization of volunteers with *E. coli* J5 did not result in a marked elevation of anti-J5 LPS antibodies, or in an elevation of anti-lipid A, anti-Re LPS, or anti-smooth LPS antibodies. This further raises the question of the mechanism of protection afforded by anti-J5 serum or plasma. One possible explanation for this discrepancy is that the antibody responsible for protection has not been accurately measured by the serologic methods used.

Detection of antibodies to the various core LPS is difficult because of the limited solubility and self-aggregating properties of hydrophobic core LPS, which may lead to nonspecific binding of antibodies and to irregular coating of ELISA plates. To avoid these problems, we modified the physicochemical state of core LPS by complexing it to HDL, a natural carrier of HDL in vivo, and used HDL-LPS complexes to coat the plates. This method has been shown to increase the sensitivity and the specificity of the detection of core LPS or lipid A antibodies [4]. In addition, the core region itself shows significant inter- and intraspecies differences in its composition [13], and type-specific antibodies are elicited after immunization with rough mutants. The structures most likely to induce broadly cross-reactive anti-LPS antibodies are the most conserved parts, that is, lipid A or the lipid A–KDO region. Therefore, if lipid A or lipid A–KDO antibodies are a subset of J5 LPS antibodies, their detection might be obscured by the quantitatively predominant type-specific J5 LPS antibodies. Moreover, since LPS in outer membranes is heterogeneous [16], the innermost region of LPS may be exposed at the surface of the whole J5 bacteria, whereas only complete LPS with core sugars may be present in the J5 LPS extracted by chemical methods. Thus, immunization with whole J5 bacteria would induce core LPS antibodies not detectable when using purified J5 LPS as antigen.

We investigated whether J5 vaccine induced the production of antibodies against lipid A or the lipid A–KDO region that would not be adequately detected by ELISA using J5 LPS as antigen. We could not detect significant increases of antibodies to two lipid A and two Re LPS extracted both from

S. minnesota R595 or from *E. coli* D31m4. Since the use of HDL-LPS complexes instead of free LPS as antigen may mask epitopes of the core LPS, it remains possible that antibodies against these hidden epitopes may be missed. This seems unlikely, however, because monoclonal antibodies directed against well-defined epitopes of core LPS easily recognize their epitopes within HDL complexes [4]. Our results question the role of antibodies to lipid A or to lipid A–KDO as protective factors in J5 antiserum, although these negative findings must be compared with those of clinical studies (now underway) with anti-lipid A monoclonal antibodies and with purified intravenous immunoglobulins enriched in anti-Re LPS antibodies.

Another possible explanation for the discrepancy between the protection afforded by J5 antiserum and the weak anti-J5 LPS antibody increase observed is that the factor responsible for the protection is a nonspecific polyclonal antibody increase rather than cross-protective anti-core LPS antibodies. Indeed, studies in rabbits suggest that a polyclonal response against O antigens may occur after intravenous immunization with J5 bacteria [17]. However, the present data suggest that this is unlikely in man, because there was neither a significant increase in antibodies to LPS from smooth gram-negative bacteria nor an increase in the total IgG or IgM serum levels. Alternative explanations may be that J5 vaccine increased nonspecifically some unrecognized acute-phase reactants capable of neutralizing LPS, altering its metabolism, or counteracting the biologic effects of humoral or cellular factors released by the stimulation of LPS. Additional studies are needed to explore these possibilities.

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