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Estimation of Protective Levels of Anti-O-Specific Lipopolysaccharide Immunoglobulin G Antibody against Experimental *Escherichia coli* Infection

DEBORAH E. SCHIFF,[1] CAROL A. WASS,[1] STANLEY J. CRYZ, JR.,[2] ALAN S. CROSS,[3] AND KWANG SIK KIM[1]*

Childrens Hospital Los Angeles, Los Angeles, California 90027[1]; Swiss Serum and Vaccine Institute, Bern, Switzerland[2]; and Department of Bacterial Diseases, Walter Reed Army Institute of Research, Washington, D.C. 20307[3]

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Serum obtained after immunization with an O18 polysaccharide-toxin A conjugate vaccine was evaluated for the estimation of protective levels of anti-O-specific lipopolysaccharide (LPS) immunoglobulin G (IgG) antibody against bacteremia and death caused by a homologous serotype of *Escherichia coli* K1 strains. Passive transfer of rabbit serum conferred significant protection from a lethal *E. coli* infection in a neonatal rat model. The overall incidence of bacteremia and mortality was 4% in rat pups receiving undiluted postvaccination serum, while that in control animals was 100% ($P < 0.001$). The overall incidences of bacteremia were 5 and 72% for animals with serum anti-O18 LPS IgG concentrations of >1.0 and <1.0 μg/ml, respectively, while the overall incidences of mortality for animals with serum anti-O18 LPS IgG levels of >1.0 and <1.0 μg/ml were 0 and 72%, respectively ($P < 0.001$). Protection against *E. coli* infection was also demonstrated with human anti-O18 polysaccharide IgG. None of the animals with human anti-O18 LPS IgG levels of >1 μg/ml had bacteremia after bacterial challenge, whereas all animals with bacteremia at 18 h had levels of <1 μg/ml. These findings suggest that serum anti-O18 LPS IgG concentrations of >1.0 μg/ml may provide protection against bacteremia and death caused by a homologous *E. coli* K1 infection.

Escherichia coli sepsis continues to be associated with an unacceptably high mortality rate, despite the availability of potent antimicrobial agents. Most human *E. coli* blood isolates fall within a small number of O serotypes; O18 lipopolysaccharide (LPS) is the serotype most frequently associated with bacteremia (3, 15, 19). Because of these findings, a prototype O polysaccharide (PS)-protein conjugate vaccine comprising O18 PS without lipid A conjugated to toxin A (TA) of *Pseudomonas aeruginosa* was developed (5, 6). This conjugate vaccine was found to be nontoxic and nonpyrogenic (6). The immunogenicity of the O18 PS-TA conjugate vaccine was previously demonstrated with an enzyme-linked immunosorbent assay (ELISA) (5, 6). In the present study, in an attempt to estimate protective levels of specific anti-O18 LPS antibody in a neonatal rat model, we correlated serum anti-O18 LPS immunoglobulin G (IgG) concentrations derived from the O18 PS-TA conjugate vaccine with in vivo protection against bacteremia and death caused by *E. coli* K1.

MATERIALS AND METHODS

Preparation of hyperimmune rabbit serum. The conjugate vaccine consisted of O18 PS conjugated to TA of *P. aeruginosa* as previously described (5). A New Zealand White rabbit (2.5 kg) was immunized intramuscularly with 50 μg of O PS (with TA as a conjugate) in 0.5 ml of phosphate-buffered saline (PBS) on days 0 and 14. Serum samples were obtained on days 0 (preimmunization) and 28.

ELISA. O18 LPS was extracted from *E. coli* 205 (O18:K nontypeable) by the hot phenol-water method of Westphal et al. (25), purified as described previously (5), and used for the ELISA. A stock solution of O18 LPS was made by dissolving 5 mg of O18 LPS in 1 ml of 36 mM triethylamine in distilled water. This solution was stored at 4°C. Polystyrene microtitration plates (Immulon; Dynatech, Buchs, Switzerland) were coated by placing in each well 100 μl of a 1-μg/ml O18 LPS solution (diluted from the stock solution) in PBS (pH 7.1 to 7.4). The coating antigen concentration was previously determined by plotting the optical density (OD) against increasing antigen concentrations. The antigen concentration that yielded the maximal OD before the plateau was selected. Antigen-coated plates were incubated for 2 h at 37°C and then overnight at 4°C. The plates were washed three times with PBS containing 0.02% Tween 20 (PBS-T) just before use. One hundred microliters of 3% bovine serum albumin in PBS was added to each well to block nonspecific antibody binding. The plates were incubated at 37°C for 1 h before being washed again with PBS-T. Sera to be tested were serially diluted (starting at a 1:10 dilution) in PBS-T, and 100 μl was added to each well. All samples were run in duplicate. Control wells without serum were included, and the control values were subtracted from the test values. The plates were incubated at 37°C for 1 h and then washed three times with PBS-T. A 1:5,000 dilution of horseradish peroxidase-coupled goat anti-rabbit IgG antibody specific for heavy and light chains (Nordic Immunological Laboratories, Tilburg, The Netherlands) in PBS-T was then added (100 μl per well), and the mixture was incubated for 2 h at 37°C. After three washes with PBS-T, 100 μl of the substrate *o*-phenylenediamine (0.4 mg/ml) in citrate-phosphate buffer (pH 5.0) was added to each well, and the mixture was incubated at room temperature in the dark for 10 min. The reaction was stopped with 100 μl of 2.5 N HCl per well. The A_{490} was read with a V_{max} kinetic microplate reader (Molecular Devices Corp., Menlo Park, Calif.).

* Corresponding author.

The total concentration of specific anti-O18 LPS IgG in the rabbit sera was determined by a modification of the procedure described by Cryz et al. (7). An affinity-purified goat anti-rabbit IgG Fc fragment-specific antibody (Jackson Immunoresearch Laboratories, West Grove, Pa.) was diluted in PBS (pH 7.1 to 7.4) to a concentration of 0.33 μg/ml, and 100 μl was added to each well. The optimal antibody coating concentration was previously determined by plotting the OD against increasing antibody concentrations and selecting the concentration yielding the maximal OD before the plateau. The plates were incubated for 2 h at 37°C and then overnight at 4°C. After three washes with PBS-T, 100 μl of reference rabbit gamma globulin (Jackson Immunoresearch Laboratories) was added to each well in duplicate after undergoing 12 twofold serial dilutions with PBS-T starting from a concentration of 5 μg/ml. Reference rabbit gamma globulin and postvaccination rabbit serum were run on the same plate by simply coating some wells with O18 LPS and other wells with goat anti-rabbit IgG. The plates were then processed in accordance with the ELISA methodology described above. A reference curve was produced by plotting the A_{490} against the log of the rabbit IgG concentration. The dilutions of rabbit serum yielding an absorbance within the linear region of the reference curve were used to calculate the concentration (in micrograms per milliliter) of specific rabbit anti-O18 LPS IgG. Once the concentration of specific rabbit anti-O18 LPS IgG was determined, the postvaccination rabbit serum was used as a standard to analyze the sera from passively immunized rat pups for micrograms of rabbit anti-O18 LPS IgG per milliliter.

ELISA conditions were modified slightly when human anti-O18 LPS was assayed. Coating concentrations of affinity-purified sheep anti-human IgG (gamma chain) (Binding Site, San Diego, Calif.) and O18 LPS IgG were both 5 μg/ml. Human serum with a known concentration of nonspecific IgG (Kent Labs Inc., Redmond, Wash.) was used as a reference. Human anti-O18 PS sera pooled after QAE/Sephadex column IgG isolation were used as a standard. A monoclonal murine anti-human IgG (Fc receptor)–peroxidase conjugate (Binding Site) was used at a 1:5,000 dilution.

ELISA specificity was demonstrated by antigen-specific inhibition. Rabbit serum from day 28 postimmunization was diluted 1:100 in PBS-T, and this dilution was mixed with twofold dilutions of inhibiting antigens (O18 LPS or O7 LPS) starting from a concentration of 10 μg/ml. The serum-antigen mixture was incubated for 2 h at 37°C and then overnight at 4°C before being added to O18 LPS-coated wells. The remainder of the ELISA was performed as described above.

Human anti-O18 PS IgG isolation. Pre- and postvaccination human anti-O18 PS sera were obtained as described previously (6). IgG was isolated by use of a QAE/Sephadex column as previously described (12). Specific anti-O18 LPS IgG levels were determined for samples before and after IgG isolation by the ELISA technique described above. In addition, total human IgG, IgM, and IgA were quantitated by use of radial immunodiffusion kits from Binding Site.

Protection studies. Outbred pathogen-free Sprague-Dawley pregnant rats with timed conception were purchased from Charles River Breeding Laboratories, Wilmington, Mass., and gave birth in our vivarium 5 to 7 days after arrival. Each adult rat and her pups were housed in a separate solid polypropylene opaque cage with a filter hood.

E. coli infection was induced in 5-day-old rats (weighing 10 g) by a previously detailed method (10). In brief, 57 5-day-old rats were immunized by an intraperitoneal injection with 0.05 ml of postvaccination rabbit serum at one of the following dilutions—undiluted, 1:2, 1:5, 1:20, 1:50, 1:100—or an equal volume of prevaccination rabbit serum or sterile saline. Twenty-four hours later, blood was obtained from the external jugular vein for the anti-O18 LPS IgG determination by the ELISA. The rats were then inoculated subcutaneously with 5×10^2 CFU of *E. coli* C5 (O18:K1). As shown previously (10), this inoculum produces nonlethal bacteremia within 18 h and death within 72 h in almost 100% of animals. At 18 h after inoculation, 50 μl of blood was obtained for quantitative culturing. Blood was diluted 20-fold in brain heart infusion broth and further diluted 10-fold in sterile saline. Twenty microliters of each dilution was spread on blood agar. The lowest dilution was also incubated overnight at 37°C, and a loopful was streaked on blood agar to detect bacteremia caused by $<10^3$ CFU/ml. Mortality was recorded for 3 days following the injection of *E. coli*, and postmortem blood culturing was done to confirm *E. coli* infection.

Additional animal protection studies were performed with another *E. coli* O18:K1 serotype strain, 221. Animals received 0.05 ml of postvaccination rabbit serum at one of the following dilutions—undiluted, 1:2, or 1:4—or an equal volume of prevaccination rabbit serum or sterile saline. At 24 h later, blood was obtained for the anti-O18 LPS IgG determination, and the rats were inoculated subcutaneously with 10^3 CFU of *E. coli* 221. This inoculum was determined by our laboratory to produce nonlethal bacteremia within 18 h and death within 72 h in 100% of animals studied. Quantitative blood culturing was performed and mortality was observed as previously described.

Studies of protection against *E. coli* C5 were also performed with isolated human anti-O18 PS IgG. Animals received 0.05 ml of human anti-O18 PS IgG diluted to a concentration of either 47, 23.5, or 2.4 μg/ml or an equal volume of preimmune IgG or sterile saline. Animals were observed for bacteremia and mortality following inoculation with *E. coli* C5.

Statistics. The statistical significance between bacteremia and mortality rates for animals grouped by anti-O18 LPS IgG antibody concentration was determined by chi-square analysis. The significance of differences between geometric mean titers (GMT) for animals grouped by presence versus absence of bacteremia or mortality versus survival was determined by use of an unpaired *t* test.

RESULTS

Results from ELISA inhibition studies with O7 LPS (heterologous) and O18 LPS (homologous) are shown in Fig. 1. Increasing concentrations of O7 LPS had no significant inhibitory effect on ELISA absorbance readings for O18 LPS, while increasing the concentration of O18 LPS to 10 μg/ml led to a 92% inhibition of ELISA absorbance readings, thereby demonstrating the specificity of the ELISA for anti-O18 LPS IgG.

Ninety six percent of total human anti-O18 LPS IgG was conserved following QAE/Sephadex column isolation, with concentrations of 49 and 47 μg/ml pre- and post-IgG separation, respectively. Sixty percent of total IgG was recovered; 1.6 and <0.4% of total IgM and IgA were recovered, respectively.

The O18 PS-TA conjugate vaccine engendered a 69-fold increase in rabbit anti-O18 LPS IgG, with pre- and postvaccination levels of 0.4 and 27.5 μg/ml, respectively. Passive transfer of postvaccination rabbit serum conferred protection against encapsulated *E. coli* K1 C5 (018) in a neonatal

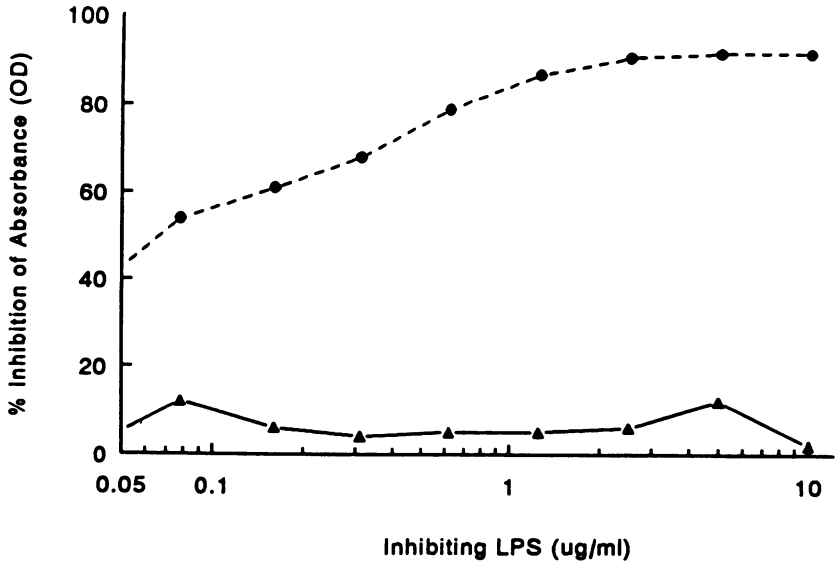

FIG. 1. Inhibition ELISA with O18 LPS (●) and O7 (▲) LPS.

rat model (Table 1). Overall mortality was 5% for rat pups receiving undiluted postvaccination rabbit serum, while mortality was 100% for rat pups receiving postvaccination rabbit serum diluted ≥1:5, undiluted prevaccination rabbit serum, or sterile saline ($P < 0.001$). All postmortem blood cultures grew *E. coli*. The levels of rabbit anti-O18 LPS IgG in sera from 38 neonatal rats were determined. Fifteen samples were not included in the antibody analysis because of a background absorbance of >0.2 unit. An additional four samples were not included in the anti-O18 LPS IgG level determination because of an insufficient quantity of sera. These samples were, however, included in the analysis of bacteremia and mortality. As expected, GMT correlated with treatment received (Table 1). Animals receiving undiluted postvaccination serum, postvaccination serum diluted 1:2, and saline had GMT of 2.0, 0.7, and 0.2 μg/ml, respectively.

TABLE 1. Protective effect of rabbit anti-O18 PS serum against *E. coli* C5 (O18:K1)

Treatment (no. of animals)	No. of animals with bacteremia (overall % bacteremia)	No. of deaths (overall % mortality)	GMT of anti-O18 LPS IgG, mean μg/ml ± SD (*n*)
Postvaccination serum			
Undiluted (19)	1 (5)[a,b]	1 (5)[a,b]	2.0 ± 0.7 (12)
Diluted 1:2 (7)	4 (57)	3 (43)	0.7 ± 0.6 (7)
Diluted 1:5 (4)	4 (100)	4 (100)	0.5 ± 0.1 (4)
Diluted 1:20 (4)	4 (100)	4 (100)	0.1 ± 0.0 (2)
Diluted 1:50 (4)	4 (100)	4 (100)	0.1 ± 0.0 (2)
Diluted 1:100 (7)	7 (100)	7 (100)	0.5 ± 0.3 (3)
Prevaccination serum, undiluted (4)	4 (100)	4 (100)	0.3 ± 0.1 (4)
Saline (8)	8 (100)	8 (100)	0.2 ± 0.2 (4)

[a] $P < 0.001$ as determined by chi-square analysis in comparison with groups receiving postvaccination serum diluted ≤1:5, prevaccination serum, or saline.

[b] $P < 0.05$ as determined by chi-square analysis in comparison with the group receiving postvaccination serum diluted 1:2.

Protection against another encapsulated *E. coli* K1 strain, 221 (O18), was also conferred by passive transfer of postvaccination rabbit serum. All animals receiving postimmune serum that was undiluted or diluted 1:2 or 1:4 survived, and no bacteremia was detected (Table 2). One hundred percent of animals that received preimmune serum or sterile saline had bacteremia and died ($P < 0.001$). The rabbit anti-O18 LPS IgG levels that were protective for strain 221 were lower than those that were protective for strain C5. None of the animals with rabbit anti-O18 LPS IgG levels of >0.2 μg/ml had bacteremia or died from infection with *E. coli* 221. These results were consistent with prior experience with these strains in our laboratory; C5 has been shown to be a more virulent strain of *E. coli* in vitro and in vivo (13).

A level of rabbit anti-O18 LPS IgG in serum of >1.0 μg/ml following passive transfer of postimmune rabbit serum was associated with protection from bacteremia and death in the neonatal rat model of *E. coli* O18:K1 serotype sepsis. The overall incidences of bacteremia were 5 and 72% for animals with anti-O18 LPS IgG concentrations of >1.0 and <1.0 μg/ml, respectively ($P < 0.001$). The overall incidences of

TABLE 2. Protective effect of rabbit anti-O18 PS serum against *E. coli* 221 (O18:K1)

Treatment (no. of animals)	No. of animals with bacteremia (overall % bacteremia)	No. of deaths (overall % mortality)	GMT of anti-O18 LPS IgG, mean μg/ml ± SD
Postvaccination serum			
Undiluted (6)	0 (0)[a]	0 (0)[a]	4.5 ± 2.09
Diluted 1:2 (6)	0 (0)[a]	0 (0)[a]	0.8 ± 0.3
Diluted 1:4 (4)	0 (0)[a]	0 (0)[a]	0.3 ± 0.1
Prevaccination serum, undiluted (4)	4 (100)	4 (100)	0.06 ± 0.004
Saline (2)	2 (100)	2 (100)	0.05 ± 0.0

[a] $P < 0.001$ as determined by chi-square analysis in comparison with the group receiving prevaccination serum or saline.

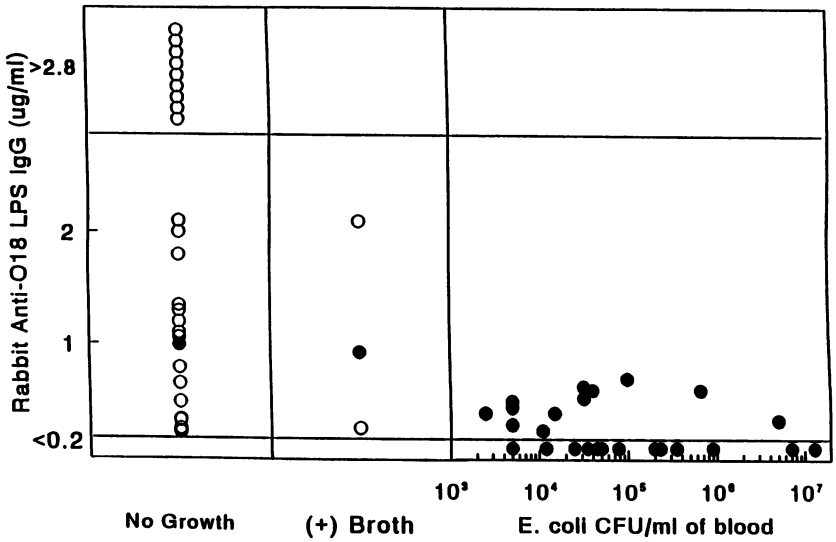

FIG. 2. Rabbit anti-O18 LPS IgG antibody levels in serum at 0 h in bacteremic and nonbacteremic rats. Symbols: ○, survived; ●, died. No Growth, cultures were negative; (+) Broth, broth was positive.

mortality for animals with concentrations of anti-O18 LPS IgG in serum of >1.0 and <1.0 μg/ml were 0 and 72%, respectively ($P < 0.001$).

Nineteen of 30 animals with sterile blood cultures done with samples obtained 18 h after inoculation with *E. coli* O18:K1 serotype strains had a concentration of rabbit anti-O18 LPS IgG of >1.0 μg/ml (Fig. 2). All animals with $>10^3$ CFU of *E. coli* per ml had a concentration of rabbit anti-O18 LPS IgG in serum of <1.0 μg/ml (Fig. 2). Survival was also correlated with the concentration of rabbit anti-O18 LPS IgG in serum. Twenty of 31 surviving animals had a concentration of rabbit anti-O18 LPS IgG of >1.0 μg/ml, while all animals with fatal *E. coli* infection had a concentration of <1.0 μg/ml (Fig. 2).

The GMT (mean ± standard deviation) of anti-O18 LPS IgG in sera of animals grouped by mortality versus survival and the presence versus the absence of bacteremia were compared. Animals with ($n = 30$) and without ($n = 30$) bacteremia had GMT of 0.3 ± 0.4 and 1.9 ± 1.8 μg/ml, respectively ($P < 0.001$; unpaired t test). Surviving animals ($n = 31$) had a GMT of 1.9 ± 1.8 μg/ml, and animals with lethal *E. coli* infection ($n = 29$) had a GMT of 0.3 ± 0.3 μg/ml ($P < 0.001$; unpaired t test).

Protection against *E. coli* O18 C5 was also demonstrated with human anti-O18 LPS IgG (Table 3). The overall incidences of bacteremia and death were 13 and 17%, respectively, for animals that received a dose of ≥1.2 μg of human anti-O18 LPS IgG, while the incidence of bacteremia and death was 100% for control animals, which received preimmune human IgG or sterile saline ($P < 0.001$). None of the animals with human anti-O18 LPS IgG levels of >1 μg/ml had bacteremia at 18 h (Fig. 3). However, two animals with levels of >1 μg/ml and negative blood cultures at 18 h subsequently died. Postmortem cultures for these animals were positive for *E. coli*. All animals with bacteremia at 18 h had levels of <1 μg/ml. Thirteen of 15 animals (87%) with anti-O18 LPS IgG levels of >1 μg/ml survived, while 7 of 21 (33%) with levels of <1 μg/ml did so ($P < 0.01$).

DISCUSSION

E. coli is the leading cause of nosocomial gram-negative sepsis (2, 17). Even with the use of broad-spectrum antibiotics and optimal supportive care, mortality rates as high as 30% have been reported (2, 17, 22, 24). Attempts have been made to develop an immunologic approach to the prevention of and therapy for *E. coli* sepsis. The feasibility of this approach is based on the facts that *E. coli* strains possessing the K1 capsular PS represent the predominant capsular type responsible for *E. coli* sepsis and that approximately 10 to 12 O serotypes account for the majority of *E. coli* blood isolates, with little geographic variation (3, 15, 18, 19). Antibodies to specific bacterial surface determinants, such as the capsular PSs and O-specific antigens, have been shown to mediate opsonophagocytosis of *E. coli* and to offer protection in animal models of *E. coli* infection (4, 9, 14, 20). Thus, both the K1PS antigen and O LPS are potential vaccine candidates. However, the K1 PS is poorly immunogenic because of antigenic relatedness to mammalian glycoproteins and glycolipids (23). Efforts to improve the immu-

TABLE 3. Protective effect of human anti-O18 PS IgG against *E. coli* C5

Treatment (no. of animals)	No. of animals with bacteremia (overall % bacteremia)	No. of deaths (overall % mortality)	GMT of anti-O18 LPS IgG, mean μg/ml ± SD
Human anti-O18 LPS IgG			
2.4 μg (14)	1 (7)[a]	3 (21)[a]	2.6 ± 1.9
1.2 μg (9)	2 (22)[a]	1 (11)[a]	1.2 ± 0.6
0.1 μg (2)	2 (100)	1 (50)	0.04 ± 0.005
Preimmune human IgG (4)	4 (100)	4 (100)	0.02 ± 0.01
Saline (7)	7 (100)	7 (100)	0.02 ± 0.02

[a] $P < 0.001$ as determined by chi-square analysis in comparison with the group receiving preimmune IgG or saline.

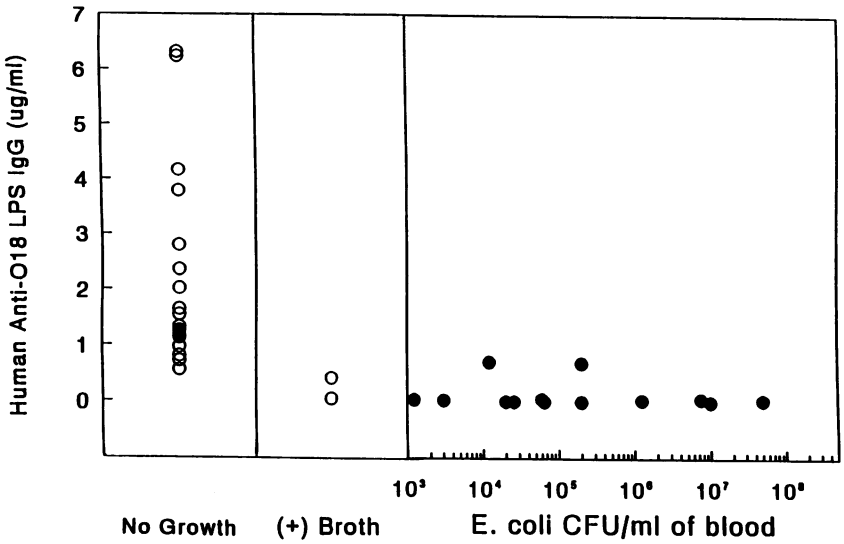

FIG. 3. Human anti-O18 LPS IgG antibody levels in serum at 0 h in bacteremic and nonbacteremic rats. Symbols: ○, survived; ●, died. No Growth, cultures were negative; (+) Broth, broth was positive.

nogenicity of the K1 PS must be pursued with caution. There is some concern that antibodies to the K1 PS may bind to host tissues and have a harmful effect. The use of anti-O LPS antibody may be an attractive alternative, since the number of O serotypes commonly isolated from blood are limited. We have shown the ability of a serospecific monoclonal anti-O18 LPS antibody to protect against experimental homologous *E. coli* K1 infections (14). This result indicates that the known protective effect of the K1 capsule can be overcome by a serospecific anti-O LPS antibody.

It is known that most individuals with *E. coli* bacteremia mount a significant antibody response, primarily directed against the O PS moiety of the LPS molecule (16). Prior to this report, however, the concentration of antibody in serum necessary to confer protection against *E. coli* infection in vivo had yet to be determined. Previously, the only gram-negative bacterium to be studied to determine protective levels of anticapsular PS antibody in serum was *Haemophilus influenzae* type b. Studies have indicated that an anticapsular PS antibody level against *H. influenzae* type b of ≥0.1 μg/ml, as measured by a radioimmunoassay, confers significant protection from *H. influenzae* type b bacteremia and meningitis in both rats and humans (1, 21).

In the present study, we showed that an O18 PS-TA conjugate vaccine was immunogenic and induced a 69-fold increase in anti-O18 LPS IgG levels in a New Zealand White rabbit. These findings are consistent with those of Cryz et al. (5). In addition, we demonstrated in vivo protection against *E. coli* infection by using passive transfer of postvaccination serum to neonatal rats. Because O18 PS-TA is a protein conjugate vaccine, we hypothesized that the immunity incurred would be T cell mediated and therefore that IgG, not IgM, would be largely induced. To determine the role of specific IgG, we isolated human IgG from postvaccination serum to exclude the possibility of a protective effect of anti-O18 LPS IgM. We determined that concentrations of rabbit and human anti-O18 LPS IgG in serum of >1.0 μg/ml effectively protected against bacteremia and death caused by homologous *E. coli* K1 C5 and 221. As previously mentioned, protective antibody levels against *H. influenzae* type b are similar for both rats and humans (1, 21). Protective anti-O LPS levels against homologous serotypes of *E. coli* need to be determined for humans.

Since this prototype serospecific O PS-protein conjugate vaccine has been found to be safe in humans (6) and since antibody induced by this vaccine confers protection against lethal *E. coli* infection (5, 6), the idea of a polyvalent serospecific *E. coli* vaccine against the most common *E. coli* O serotypes is an attractive one. A promising use for such a vaccine would be to prepare hyperimmune gamma globulin for intravenous use (IVIG) from the plasma of immunized healthy donors. We have previously shown that the administration of a monoclonal anti-O-specific antibody in conjunction with an antibiotic significantly improves the survival of animals with experimental *E. coli* infection (9), suggesting that antibodies directed to O LPS may be a useful adjunct to antimicrobial chemotherapy for *E. coli* infection. By use of hyperimmune IVIG, a larger quantity of functional anti-O LPS antibody can be administered in a comparatively small volume, thus circumventing some of the adverse reactions that are occasionally noted when large volumes of nonhyperimmune IVIG are given (11).

Monospecific IVIGs have already been developed against *P. aeruginosa* and *Klebsiella* species (8). Because most fatal nosocomial infections caused by gram-negative bacilli are caused by *E. coli*, *P. aeruginosa*, and *Klebsiella* species, a polyvalent IVIG to the above-mentioned pathogens would increase the spectrum of activity and allow for use in a prophylactic or therapeutic mode. High-risk patients with poor host defense, such as prematurely born infants and patients undergoing immunosuppressive chemotherapy, may ultimately benefit from the use of a polyvalent IVIG preparation to the gram-negative bacteria most commonly implicated in gram-negative sepsis.

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