Induction of Immunity against Lethal *Haemophilus influenzae* Type b Infection by *Escherichia coli* Core Lipopolysaccharide

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ABSTRACT Efforts to prevent *Haemophilus influenzae* type b (HIB) infections in infancy have been hampered by the low immunogenicity of capsular polysaccharide vaccines in children younger than 18 mos. In searching for alternate immunogens, we have studied the protective potential of polysaccharide-poor, lipid-rich endotoxin (LPS) core in experimental HIB infections. Because all gram-negative bacteria have similar LPS core structures, we were able to use as vaccine the J5 mutant of *Escherichia coli* 0111, the LPS of which consists only of core components, and thus to avoid problems in interpretation arising from vaccine contamination with non-LPS HIB immunogens. Mice were given graded inocula of HIB and developed lethal infection analogous to human HIB disease when virulence was enhanced with mucin and hemoglobin. After active immunization with heat-killed *E. coli* J5, 40/50 (80%) of infected mice survived, compared with 14/50 (28%) of saline-immunized controls ($P < 0.005$). Passive immunization with rabbit antiserum against *E. coli* J5 prevented lethal HIB infection when administered 24 or 72 h before or 3 h after infection. This protection was abolished by adsoprtion of antiserum with purified J5 LPS, with survival reduced from 14/24 to 0/24 ($P < 0.005$). Furthermore, rabbit antiserum to purified J5 LPS gave just as potent protection against death as antiserum to whole J5 cells. These studies demonstrate that immunity to core LPS confers protection against experimental murine HIB infection and provide the framework for a new approach to prevention of human disease from *HIB*.

INTRODUCTION

Haemophilus influenzae type b (HIB)[1] is a major cause of bacteremia, meningitis, arthritis, and pneumonia in children. Although antibiotics have reduced mortality, these infections continue to exact a high toll in the form of acute complications and long-term neurologic sequelae (1). HIB capsular polyribose phosphate vaccine has failed to prevent these infections in children <18 mo of age (2). Recent evidence supporting the role of subcapsular components in resistance to HIB infections (3–5) led us to investigate the possibility that vaccination with one of these components, lipopolysaccharide (LPS), would stimulate protective immunity against HIB infection.

The LPS immunogen we chose to study is the J5 mutant of *Escherichia coli* 0111:B_4, a rough mutant lacking both the enzyme uridine 5′-diphosphate galactose 4-epimerase and the ability to incorporate exogenous galactose into its LPS. The epimerase deficiency prevents attachment of oligosaccharide sidechains to LPS core (6), and the defect in galactose incorporation confers phenotypic stability. *E. coli* J5 has several special properties which make it an appealing candidate vaccine against HIB: (*a*) LPS from the J5 mutant contains only core glycolipid determinants (lipid A, *N*-acetylglucosamine, 2-keto-3-deoxyoctonate, heptose, and glucose). Core regions of LPS from a wide variety of enteric bacilli are structurally related (7). Lipid A from LPS of several enteric and nonenteric gram-negative bacteria including *H. influenzae* are antigenically similar (8). Use of common core LPS vaccine from *E. coli* rather than HIB elim-

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[1] *Abbreviations used in this paper*: BHI, brain heart infusion; CFU, colony-forming units; HA, hemagglutinating antibody; LD_{50}, median lethal inoculum; LPS, lipopolysaccharide; Muc/Hgb, mucin/hemoglobin.

inates problems of vaccine contamination with other HIB components so that the importance of immunity to LPS alone can be assessed. (*b*) Antibody against J5 core LPS protects animals against LPS from *E. coli*, *Salmonella typhimurium*, and all three major serogroups of *Neisseria meningitidis* (9–11) and against lethal bacteremia due to *E. coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (12, 13). (*c*) J5 boiled bacterial vaccine has been administered safely to a large number of adult human subjects without complication, and the human serum obtained after J5 immunization protects animals and man against death from gram-negative bacteremia (13, 14).

To demonstrate protection by *E. coli* J5-induced immunity we used an experimental mouse infection in which bacteremia, brain infection, and high mortality resemble human HIB disease. Passive protection was abolished after adsorption of J5 boiled cell antiserum with purified J5 LPS, and was duplicated using antiserum made by immunization with purified J5 LPS. These results offer promise that core LPS antigens may enhance immunogenicity and protection of vaccines to prevent human HIB infection.

METHODS

Bacteria. Three recent isolates of *HIB* were obtained from the cerebrospinal fluid or blood of children with meningitis seen at the Montreal Children's Hospital. Strain 579 produced β-lactamase; strains 544 and 586 did not. HIB was maintained by daily passage on Levinthal's agar (brain heart infusion [BHI], 36.4 g/liter; proteose peptone No. 3, 44.0 g/liter; agar, 16.0 g/liter; and Fildes enrichment, 100 ml/liter—all from Difco Laboratories, Detroit, MI) incubated at 37°C in 5% CO_2. Two strains of *E. coli*, HIB 7 and HIB 299, which contain the K100 capsular antigen cross-reactive with HIB capsular polyribose phosphate, were obtained from Dr. George McCracken Jr., University of Texas, Southwestern, Dallas, TX. *S. typhimurium* strain ATCC 9800 was obtained from the American Type Culture Collection, Rockville, MD. *E. coli* 0111:B_4 and its uridine 5′ = diphosphate galactose 4-epimeraseless mutant, *E. coli* J5, was obtained in 1966 from Dr. Edward Heath, Johns Hopkins Hospital, Baltimore, MD. From cultures of *E. coli* J5 we have selected a strain which differs from the original mutant in that it can no longer incorporate exogenous galactose into its LPS. This J5 strain has been stable for >15 yr on trypticase soy agar (Difco Laboratories) slants at 25°C. Before each use the enteric bacilli were subcultured on blood agar at 37°C.

Extraction of core LPS. LPS was prepared from *E. coli* J5 by the phenol-chloroform-petroleum ether technique of Galanos et al. (15).

Preparation of rabbit antisera. E. coli J5 was grown in trypticase soy broth (Difco Laboratories) overnight at 37°C and HIB strains were grown in Levinthal's broth (BHI, 36.4 g/liter; proteose peptone No. 3, 44.0 g/liter; Fildes enrichment, 50 ml/liter—all from Difco Laboratories) overnight at 37°C in 5% CO_2. Cells were harvested by centrifugation, washed three times in 0.15 M NaCl, and boiled for 2.5 h. After boiling the cells were resuspended in 0.15 M NaCl and adjusted spectrophotometrically to a concentration of 5 × 10^9 cell/ml. Boiled cell antiserum to *E. coli* J5 or HIB was produced in 3-kg New Zealand White rabbits (Hills of Home Ranch, Jamul, CA) by nine intravenous injections of vaccine over 3 wk in gradually increasing doses (0.1, 0.3, 0.5, 0.5, 0.7, 0.9, 0.9, 1.0, and 1.0 ml). Antiserum to purified core LPS was prepared by injecting J5 LPS into rabbits twice weekly for 3 wk. The LPS was well-suspended in 0.15 M NaCl and given intravenously in gradually increasing doses 0.05, 0.1, 0.2, 0.5, 0.5, and 0.5 mg). Antisera were collected 7 d after the final injection and were stored at −20°C along with the nonimmune serum collected from the same rabbits before immunization.

To enrich for capsular antigens, overnight cultures of HIB or *E. coli* K100 strains were killed in 1% formaldehyde, then harvested, washed three times in 0.15 M NaCl, and resuspended at 1 × 10^9 cells/ml. Antisera were prepared by giving rabbits six intravenous injections of these vaccines over 2 wk (0.5 ml in the first injection, 0.1 ml in the remaining five). Sera were collected 7 d after the last injection and stored at −20°C until use for in vitro studies.

Antibody determinations. Hemagglutinating antibody (HA) to core LPS was measured with human group O erythrocytes sensitized with alkaline-treated J5 LPS (16). HA to HIB capsular antigens was estimated by the method of Turk and Green (17) using 48 h Levinthal's broth culture supernatants. Immunodiffusion studies were performed in Noble agar (10 gm/liter, Difco Laboratories) at 4°C. Bactericidal antibody activity of fresh rabbit J5 antiserum was measured by a modification of the Fothergill assay (18). Possible cross-reactions between *E. coli* J5 and the two *E. coli* K100 strains were sought by inoculating molten Noble agar containing 100 ml antibody/liter with 10-fold dilutions of broth cultures, incubating the pour plates overnight at 37°C, and observing them for several days at 4°C for development of haloes of precipitation around bacterial colonies.

Removal of core LPS antibody from J5 boiled cell antiserum. Formalin-treated human group O erythrocytes were sensitized with alkaline-treated J5 LPS at a ratio of 4 mg LPS/ml packed erythrocytes according to a modification of the technique of Galanos et al. (19). The sensitized erythrocytes were then added to either J5 antiserum or nonimmune serum at a ratio of 0.1 ml packed cells/ml serum and the mixture was gently stirred at 25°C for 1 h. After centrifugation, the adsorption was repeated and the serum again separated and stored for protection experiments. To estimate the amount of contaminating LPS in these sera, one adsorption was performed using J5 LPS radiolabeled with $Na_2Cr^{51}O_4$ (Amersham Corp., Arlington Heights, IL) by a standard procedure (20).

Production of HIB infection in mice. The 6–8-wk-old female CF1 mice (Charles River Breeding Laboratories, Wilmington, MA) used for these experiments were maintained at 20–23°C at 50% relative humidity on a 7 a.m.–7 p.m. light schedule with food and water ad lib. Before each experiment HIB was mouse-passed twice in the following way. HIB strains grown overnight on Levinthal's agar were suspended in BHI and adjusted spectrophotometrically to contain 1 × 10^9 colony-forming units (CFU)/ml, then mixed in equal volume with an aqueous suspension Muc/Hgb of porcine gastric mucin, 150 g/liter (type II mucin, Sigma Chemical Co., St. Louis, MO) and bovine hemoglobin, 40 g/liter (Difco Laboratories). Mice were given intraperitoneally 1.0 ml of this mixture and 6 h later heart blood was removed aseptically for overnight culture on Levinthal's agar. For protection studies, HIB passed the previous day was grown in 50 ml Levinthal's broth on a rotary shaker at 37°C for 6 h. Serial 10-fold dilutions of the culture in BHI

were then mixed with equal volumes of Muc/Hgb and 1.0 ml of these graded inocula were given to mice intraperitoneally. All inocula were verified by colony counts. Animals were observed daily until 3 d after the last death and the median lethal inoculum (LD_{50}) was calculated by the method of Reed and Muench (21). In each experiment, survival rates were also expressed as a fraction of the total number of mice receiving serial dilutions of HIB. Several additional experiments were performed in which animals were killed to document the kinetics of the infection by quantitative tissue cultures and histologic sections of peritoneum, blood, and brain.

Active immunization of mice against lethal HIB infection. Female CF1 mice aged 5–6 wk were immunized with three-weekly subcutaneous injections of 1 ml of the following heat-killed bacterial vaccines prepared as previously described: *E. coli* J5 or HIB 579 at a concentration of 5×10^9 cells/ml; *E. coli* 0111:B_4 or *S. typhimurium* at a concentration of 1×10^9 cells/ml. All groups were challenged with HIB 579 7 d after the last immunization.

Passive immunization of mice against lethal HIB infection. Mice were given 1.0 ml of rabbit antiserum or nonimmune serum either intravenously or subcutaneously. All animals given intravenous serum were challenged with HIB 24 h later. Animals receiving serum subcutaneously were challenged with HIB either 3 h before or 24 or 72 h after serum injection. Serum for intravenous use was heated at 56°C for 30 min to eliminate interspecies hypersensitivity reactions. It was not necessary to heat serum for subcutaneous injection, and heating did not alter its potency.

Statistical methods. For each protection experiment differences among groups were examined in two ways; by comparing the LD_{50} of HIB by Student's *t* test for unpaired groups and by comparing the total survival rates by the chi-square method. The significance of differences in LD_{50} was confirmed by probit analysis, kindly performed by Dr. Robert Corbeil, University of San Diego.

RESULTS

Experimental HIB infection in mice. Combining the bacterial inoculum with mucin and hemoglobin modified the natural resistance of mice to Haemophilus enough to stimulate the high mortality observed in untreated human HIB infections. Table I shows the influence of mucin and hemoglobin, alone, and in combination, on the virulence of HIB strain 579. Addition of Muc/Hgb had an identical effect on the other two HIB strains, lowering the LD_{50} from 10^9 to 10^{2-3} CFU.

Soon after intraperitoneal injection of HIB in Muc/Hgb, mice appeared ill (lax, anorectic, immobile). As their condition worsened, tachypnea, ruffling of fur, and hypothermia developed. Moribund animals often exhibited hind leg paralysis and convulsions. Deaths were first observed 8–12 h after inoculation and they continued to occur for ~3 d. Bacteremia developed within 1 h of inoculation. Concentrations of HIB in peritoneal fluid and blood increased ~100-fold during the first 9 h and reached 10^{7-8} CFU/ml by 12 h. Peritoneal fluid contained many neutrophils and macrophages and histologic sections of peritoneum showed focal peritonitis. Brains of animals moribund at 24 h contained 10^2–10^5 CFU HIB/g tissue and showed focal, mild, mononuclear cell proliferation and edema with rare neutrophils. In survivors bacteremia cleared within 4 d but peritoneal infection persisted for 1 wk. Survivors killed at 1 wk had sterile brain cultures.

TABLE I

Enhancement of Virulence of Intraperitoneally Injected HIB Strain 579 in CF1 Mice by Mucin Alone and Combined with Hemoglobin

Vehicle for inoculum*	LD_{50} (log_{10} CFU)‡
Levinthal's broth§	9.3
BHI broth‖	>6.7
Hemoglobin, 40 g/liter	7.8
Mucin, 150 g/liter	5.0
Muc/Hgb¶	3.1

* Serial 10-fold dilutions in HBI of an overnight Levinthal's broth culture were mixed in equal volume with each vehicle and 1.0 ml given to each mouse.

‡ Each vehicle was tested in 60 mice.

§ Promotes growth of HIB.

‖ Supports viability but does not promote growth of HIB.

¶ 150 g and 40 g/liter, respectively.

Antibody determinations. J5 antisera raised by immunization of rabbits with J5 boiled cells contained HA to J5 LPS in titers of 1:1,024–1:2,048 but no antibody to erythrocytes sensitized with capsule-rich HIB 579 culture supernatants. Freshly obtained J5 antiserum had no complement-mediated bacteriolytic activity against HIB 579. *E. coli* J5 cultures diluted in agar containing antibody to the two *E. coli* K100 strains showed no precipitin haloes although all of the homologous controls were positive. In immunodiffusion studies there were no cross-reactions between J5 LPS (1 mg/ml) and antiserum to either boiled HIB cells or formalin-killed HIB cells nor between J5 antiserum to either HIB vaccine preparation.

J5 antiserum prepared by immunization with purified J5 LPS had a J5 HA titer of 1:512.

Antiserum to HIB 579 formalin-killed cells (prepared for in vitro studies) had an HA titer of 1:256 against capsule-rich HIB 579 antigens. HIB 579 boiled cell antiserum (prepared for protection studies) had a much lower anticapsular antibody titer, 1:8. Neither HIB antiserum contained HA to J5 LPS. The J5 HA titer of all lots of nonimmune serum was 1:2.

Active immunization of mice against lethal HIB infection. To determine whether any protective cross-reactivity existed between *E. coli* J5 and HIB we first vaccinated mice with J5 boiled cells, using several other gram-negative boiled cell vaccines for comparison, and saline injections as control. These mice were challenged with HIB 579 1 wk after the last vaccine injection (Table II). Protection by J5 vac-

TABLE II

*Effect of Active Immunization with Heat-killed Bacterial Vaccines on Survival of Mice Challenged with Live HIB Strain 579**

Vaccine	Experiment 1			Experiment 2		
	LD_{50} (log_{10} CFU)	Survivors/Total‡		LD_{50} (log_{10} CFU)	Survivors/Total‡	
			%			%
Saline	0.8	6/25	(24)	1.5	8/25	(32)
E. coli J5	3.3	18/25	(72)§	4.5	22/25	(88)§
HIB 579	2.8	16/25	(64)§	3.1	17/25	(68)‖
E. coli 0111:B_4	—	———		2.1	12/24	(50)¶
S. typhimurium	—	———		1.5	9/25	(36)¶

* Serial inocula 3×10^1–3×10^5.

‡ *P* values are in comparison with saline-immunized controls.

§ $P < 0.005$.

‖ $P < 0.01$.

¶ $P > 0.1$.

cine was equivalent to that seen with homologous HIB vaccine and significantly ($P < 0.005$) greater than that afforded by either saline or its parent *E. coli* 0111, whose core is encumbered by oligosaccharide side-chains. Survival after vaccination with *S. typhimurium* or *E. coli* 0111 was statistically no better than survival after saline injection. These data exclude non-specific stimulation as the mechanism of protection with J5 vaccine and highlight the importance of naked LPS core as the essential protective determinant.

Passive immunization of mice against lethal HIB infection. To assess the role of humoral immunity alone, we next studied the protective effect of passively administered J5 boiled cell antiserum on the outcome of HIB infection in mice. Survival of mice given 1.0 ml J5 antiserum intravenously 24 h before inoculation was greatly improved over that in mice given nonimmune serum (Table III). The degree of protection was identical against all three strains ($P < 0.001$) and not different ($P > 0.05$) from that observed with homologous HIB antiserum. Table IV shows that subcutaneously administered J5 antiserum was also effective when given either 24 or 72 h before inoculation. The effect of homologous HIB antiserum given subcutaneously was not statistically significant at 72 h. Heat inactivation of serum at 56°C for 30 min and given subcutaneously did not alter its potency; therefore, protection is not due to passively administered rabbit complement.

In a more rigorous test of its potency, 1.0 ml of J5 antiserum was given 3 h after HIB inoculation, when infection was already established and animals given high inocula were bacteremic. Nonimmune serum was not protective nor was physiologic saline (given as a control for the potential beneficial effect of volume expansion), but J5 antiserum gave a marked increase in survival (Table V). This therapeutic effect was not significantly different ($P > 0.1$) from the prophylactic effect reported above.

Identification of core LPS as the protective im-

TABLE III

*Prevention of Lethal HIB Infection in Mice by 1 ml i.v. Rabbit Antiserum 24 h before Inoculation**

HIB strain	Nonimmune serum			J5 antiserum			HIB 579 antiserum		
	LD_{50} (log_{10} CFU)	Survivors/Total		LD_{50} (log_{10} CFU)	Survivors/Total		LD_{50} (log_{10} CFU)	Survivors/Total	
			%			%			
579‡	2.4	17/80	(21)	4.2	47/80	(59)§	5.3	24/33	(73%)‡
544	1.7	6/20	(30)	3.5	15/20	(75)§	—	———	
586	2.2	1/15	(7)	4.2	11/15	(73)§	—	———	

* Serial inocula 2.2×10^1–2.2×10^6 HIB/ml.

‡ β-Lactamase producer.

§ In comparison with nonimmune serum controls, $P < 0.001$.

TABLE IV

*Prevention of Lethal HIB (Strain 579) Infection in Mice by 1 ml Rabbit Antiserum Given Subcutaneously**

Time before serum inoculation	Nonimmune serum			J5 antiserum			HIB 579 antiserum		
	LD_{50} (log_{10} CFU)	Survivors/Total		LD_{50} (log_{10} CFU)	Survivors/Total‡		LD_{50} (log_{10} CFU)	Survivors/Total‡	
h			%			%			%
24	4.5	28/60	(47)	>5.5	49/59	(83)§	>5.5	28/29	(97)§
72	3.3	8/30	(27)	4.7	16/30	(53)‖	4.4	13/30	(43)¶

* Serial inocula 7.5×10^2–7.5×10^5 HIB/ml.

‡ *P* values in comparison with nonimmune serum controls.

§ $P < 0.001$.

‖ $P < 0.05$.

¶ $P > 0.1$.

munogen in J5 boiled cell vaccine. The safest and most effective way to immunize animals and man with LPS is to use boiled whole bacterial cells. Heating degrades many of the surface structures but leaves LPS intact and firmly attached to the cell stroma, which acts as an excellent adjuvant. However, there is no doubt that these are complex vaccines. To be sure that the protective immunogen in J5 boiled cell vaccine is core LPS, we conducted two types of experiments. First, we tested the potency of J5 boiled cell antiserum from which J5 LPS antibody had been removed completely. Then, we evaluated the efficacy of antiserum obtained by immunization with purified J5 LPS.

We found that most conventional adsorption methods were unsatisfactory because of large quantities of residual LPS, enough in most cases so that 1.0 ml of serum given intravenously to a rabbit caused severe hypotension. We chose to use the formalinized erythrocyte method because studies using ^{51}Cr-labeled J5 LPS showed the least LPS contamination (11.7 μg/ml) in adsorbed J5 and nonimmune sera. This was still enough LPS, however, to elicit a pronounced pyrogenic response in rabbits (fever of 4°C 3 h after intravenous injection of 1.0 ml of serum). Unadsorbed J5 antiserum is never pyrogenic. Table VI shows that adsorption of J5 boiled cell antiserum with J5 LPS that lowered the J5 LPS HA titer to that of nonimmune serum and completely abolished protection against HIB infection. The fact that there were no survivors in the adsorbed J5 antiserum group suggests that contaminating LPS may have exerted a toxic effect, even though mice are more resistant to LPS than rabbits. A group of mice was given adsorbed nonimmune serum to control for nonspecific LPS-induced enhancement of resistance to infection and other stresses (22).

Although it is not practical to consider immunization of humans with purified LPS intravenously or by any other route, the limitations of adsorption experiments led us to hyperimmunize rabbits intravenously with J5 LPS. Only a few of the animals so immunized responded with HA titers to match those obtained with boiled cells. We pooled these sera and used the pool in protection experiments. Table VII shows that in two separate experiments protection against HIB infection by J5 LPS antiserum was similar to that of J5 boiled cell antiserum.

TABLE V

*Treatment of HIB (Strain 579) Infection in Mice with 1 ml Rabbit J5 Antiserum Given Subcutaneously 3 h after Inoculation**

	LD_{50} (log_{10} CFU)	Survivors/Total		
			%	
J5 antiserum	2.8	19/50	(38)	$P < 0.001$
Nonimmune serum	<1.1	1/50	(2)	

* Serial inocula 1.3×10^1–1.3×10^6 HIB/ml.

DISCUSSION

Despite the demonstrated immunogenicity and efficacy of HIB polyribophosphate vaccine in preventing HIB infections in children over the age of 2 yr, efforts to protect younger infants have not yet been successful (23, 24). Since most serious invasive HIB infections occur between the ages of 6 mo and 2 yr, we decided to explore the possibility that HIB LPS may play a role in the pathogenesis and immunity of these infections. This antigen was chosen because of its role in the pathogenesis of gram-negative bacterial infections and because immunity directed against LPS can protect against the lethal effects of some of these infections (12–14, 25, 26). Furthermore, study of the ontogeny

TABLE VI

*Effect of Removing Antibody to J5 LPS from J5 Antiserum on Protection of Mice against HIB (Strain 579) Infection**

Rabbit serum‡	HA titer	LD_{50} (log_{10} CFU)	Survivors/Total	
				%
Nonimmune serum	1:2	1.95	5/24	(21)
J5 antiserum	1:1,024	3.63	14/24	(58)§
J5 antiserum adsorbed with J5 LPS	1:2	1.63	0/24	
Nonimmune serum adsorbed with J5 LPS	1:2	1.63	0/24	

* Serial inocula 4.3×10^1–4.3×10^4.

‡ 1 ml given intravenously 24 h before inoculation.

§ Survival rate is better than with nonimmune serum ($P = 0.008$).

of immune responsiveness in mice indicates that T-independent antigens, such as LPS, are immunogenic soon after birth and 1–2 wk before polysaccharide antigens (27). If these findings can be extrapolated to humans, LPS-containing vaccines may stimulate better protection in young infants. Although we recognize the potential for toxicity with this immunogen, LPS-containing vaccines, such as pertussis and typhoid, have been used in the past with some success. Should the role of LPS be judged critical, efforts can be directed at reducing attendant toxicity.

E. coli J5 was chosen because it is a readily available and well-characterized source of core glycolipid and has previously been shown to confer cross-protective effects against diverse gram-negative bacteria and endotoxins (9–14). The mouse model was used because it provides a highly lethal bacteremic infection with meningitis and, in addition, large numbers of animals can be tested. Although the intranasal route of infection parallels events that occur in human infection more closely than the intraperitoneal route, intranasal inoculation of infant rats with HIB results in low mortality (28). This does not resemble human disease, in which untreated children with HIB meningitis die. Since it is possible that the resistance of rats to lethal infection may be due to insensitivity to the toxic effects of LPS, the rat model may not be suitable for the study of LPS-induced protection (26). Mucin-enhanced HIB infection in mice was used in the preantibiotic era to assay the potency of rabbit antisera for the treatment of human HIB meningitis (29). The mucin model has also been used for recent vaccine studies (30). Intra-

TABLE VII

Passive Immunization against HIB (Strain 579) in Mice with Antiserum to J5 LPS

Serum*	LD_{50} (log_{10} CFU)	Survivors/Total		*P* value‡
			%	
Experiment 1§				
Nonimmune serum	1.78	0/13		
J5 whole cell antiserum	2.65	8/13	(62)	<0.01‖
J5 LPS antiserum	2.41	9/13	(69)	<0.005‖
Experiment 2¶				
Nonimmune serum	2.18	17/28	(61)	
J5 whole cell antiserum	3.18	24/28	(86)	0.036
J5 LPS antiserum	3.26	25/28	(89)	0.013

* 1 ml given intravenously 24 h before inoculation.

‡ Compared with nonimmune serum.

§ Serial inocular 1.2×10^1–1.2×10^5.

‖ Yates correction for small cell frequencies.

¶ Serial inocular 3×10^1–3×10^4.

peritoneal infection is followed quickly by bacteremia, and death is consistently associated with brain infection. Corbeil et al. (31) introduced the technique of virulence enhancement by hemoglobin for the study of disseminated gonococcal infection in mice. Hemoglobin seems to have a similar effect on murine HIB infections. It is likely that the combination of mucin and hemoglobin impairs host immunity by disturbing both macrophage function and complement activity, although these mechanisms have not been worked out completely for HIB infections (32). The model results in high mortality and permits the use of small inocula, with in vivo multiplication and invasion. Inconsistent mortality rates can be explained in part by the steep dose-response curve. Hence, inaccuracies in dilutions, quantitative bacterial measurements, time of injections, and host variations from day to day may be responsible for differences in mortality between experiments. This pitfall is counterbalanced by the use of graded inocula and the inclusion of appropriate control groups in each experiment.

Our results demonstrate considerable protection against the lethal effects of HIB infection by *E. coli* J5-stimulated immunity. The effectivness of passive immunization suggests that humoral immunity is most important. The adsorption experiments and the protection afforded by antiserum prepared against purified J5 LPS lead us to believe the LPS core is the protective immunogen. The antibody class responsible for immunity, its duration, and the role of cell-mediated components remain to be defined.

Our preliminary attempts to demonstrate homology between J5 core LPS and HIB LPS have been unsuccessful. This can be explained, in part, by the fact that antibody prepared against HIB cells react most avidly with intact oligosaccharide side-chains present in late log phase and stationary phase cultures. Recently, we have further characterized the chemical and biologic properties of these two LPS and we are studying the immunologic similarities using intact and rough LPS preparations (33). Perhaps these studies will define similarities that explain the cross-protection we have observed here. Our preliminary results demonstrate both similarities and differences in selected chemical and immunogenic properties of *E. coli* J5 LPS and HIB LPS. Other attempts to characterize the biologic and chemical properties of Haemophilus LPS have also yielded conflicting results due, in part, to differences in growth procedures, bacterial strains, and extraction methods (34, 35). Our LPS was extracted by the Westphal method from HIB grown in chemically defined medium, and contained 2-keto-3-deoxyoctonate. Thus far, both our preparation and results resemble those reported by Flesher and Insel (34). Differences in biologic properties between enterobacterial LPS and other nonenteric gram-negative bacterial LPS have also been previously demonstrated for *Brucella abortus* (36) and *Pseudomonas aeruginosa* (37).

These studies prove that humoral immunity to LPS core can prevent and treat experimental murine infections with HIB. We are now stimulated to pursue further the role of LPS and other subcapsular components in the pathogenesis and immunity of HIB infection.

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