Comparative analysis of *Haemophilus influenzae* type b and *Escherichia coli* J5 lipopolysaccharides

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Summary. *Haemophilus influenzae* type b (HIB) and *Escherichia coli* J5 (J5) lipopolysaccharides (LPS) were examined to explore the basis of previously observed cross-protection. HIB-LPS and J5-LPS contained ketodeoxyoctonate, glucose, glucoheptose and glucosamine as common carbohydrate moieties, and laurate, myristate, β -hydroxymyristate and palmitate as common fatty acids, although in different ratios. J5-LPS was five times more lethal than HIB-LPS for chick embryos. Weak serological cross-reactivity was observed by haemagglutination and two-dimensional immunoelectrophoresis. No significant cross-reactivity was demonstrated by enzyme-linked immunosorbent or toxicity-neutralisation assays. The cross-reactivity observed between HIB-LPS and J5-LPS was probably due to common components in the core glycolipid.

Introduction

Haemophilus influenzae type b (HIB) is the leading cause of bacterial meningitis in young infants in the USA; 80% of the cases occur in infants under 24 months of age (Smith, 1981). Because capsular polysaccharide vaccines have been ineffective in young infants (Parke *et al.*, 1977; Peltola *et al.*, 1977) and the pathogenesis of these infections is incompletely understood, we have studied subcapsular antigens, particularly lipopolysaccharide (LPS), which might contribute to virulence and stimulate protective immunity.

Although gram-negative bacteria are heterogeneous with regard to the O antigens of LPS, the core region of this molecule appears to be similar in most species (Lüderitz *et al.*, 1966). For this reason, Re and Rc mutants have been studied as possible immunogens to protect against various gram-negative infections. The Rc mutant of *Escherichia coli* O111:B4, designated J5, has been studied as a possible vaccine against heterologous organisms, such as *Klebsiella* and *Pseudomonas* (Ziegler *et al.*, 1973, 1975 and 1982). The J5 mutant, first described by Elbein and Heath (1965), lacks the enzyme uridine diphosphate galactose 4-epimerase, which incorporates galactose into LPS, thus preventing attachment of oligosaccharide chains.

Marks *et al.* (1982b) prevented death from HIB infection in a mouse model (Marks *et al.*, 1982a) by

active immunisation with whole cells of *E. coli* J5 and by passive immunisation with the corresponding antiserum. The passive protection was abrogated by absorption of the antiserum with J5-LPS. The basis for this cross-protection is believed to be due to biochemical and immunological similarities between HIB-LPS and J5-LPS. The purpose of this investigation was to examine some of these properties and the mechanism whereby heterologous *E. coli* (J5) LPS prevents death due to experimental HIB infection.

Materials and methods

Bacteria

Haemophilus influenzae type b strain 579, a β -lactamase producer, was isolated from the cerebrospinal fluid of a child with meningitis and maintained frozen at -70°C . *Escherichia coli* J5, maintained on Trypticase Soy Agar (TSA) slants at 25°C , was derived from a strain originally obtained from Dr E. Heath, Johns Hopkins Hospital, Baltimore, MD and generously provided by Dr A. I. Braude, San Diego, CA.

Isolation and purification of lipopolysaccharides

HIB-LPS was extracted by the method of Westphal and Jann (1965) from cells grown on chocolate agar. J5-LPS was extracted by the method of Galanos *et al.* (1969) from cells grown on TSA. The final products were washed with phosphate-buffered saline (PBS) by ultracentrifugation and resuspended in PBS. For biochemical determina-

tion, LPS samples were further purified by treatment with RNAase, DNAase, and trypsin (1 mg/10 mg of carbohydrate). LPS (5–10 mg) was then applied to a 1.6 cm $\times$ 50 cm Sepharose C1-6B column and eluted with 10 mM EDTA-25 mM Tris buffer, pH 7.5. The column was calibrated with phenol red (mol. wt 1000) and dextran blue (mol. wt 2×10^6). Alternate fractions were assayed for protein and total carbohydrate. Fractions containing LPS eluted in the void volume, confirmed by fused rocket-immunoelectrophoresis with specific rabbit LPS antiserum. These fractions were pooled and dialysed against three changes of distilled water to remove EDTA-Tris.

Biochemical determinations

Total carbohydrate was measured by the phenol-sulphuric acid method of Dubois *et al.* (1956) with dextrose as the standard. Absorbance was determined at 485 nm and concentration was determined from a standard curve. Protein content was measured by the method of Bradford (1976) with the Bio-Rad Protein Assay kit (Bio-Rad, Richmond, CA). Concentration of protein was estimated from a standard curve, with bovine γ -globulin (Bio-Rad) as the standard. The amount of ketodeoxyoctonate (KDO) in LPS was determined by the method of Weissbach and Hurwitz (1959) as modified by Osborn (1963). Absorbance of the thiobarbituric acid chromophores was determined at 548 nm and the concentration determined from a standard curve with KDO (Sigma, St Louis, MO) as the standard. The presence of DNA in the LPS samples was determined by the method of Burton (1956). Absorbance was measured at 600 nm and concentrations were determined from a standard curve with DNA (*E. coli* strain B, Sigma) as the standard.

Gas-liquid chromatographic analysis of carbohydrate and fatty acids

All analyses were performed in a Hewlett Packard 5880A gas chromatograph equipped with a flame ionisation detector. Analysis of carbohydrates present in LPS preparations was performed by conversion of sugars to alditol acetate derivatives (Sawardeker *et al.*, 1965; Griggs *et al.*, 1971) with some modifications. Each sample was hydrolysed with 0.2N HCl for 72 h at 100°C. The HCl was removed by rotary evaporation and 0.5 ml of 0.6N sodium borohydride (Sigma) in 1N NH_4OH was added and stirred at room temperature for 1 h. Acetic acid was added drop by drop to destroy excess NaBH_4 and the samples dried by rotary evaporation. Methanol containing acetic acid 0.5% was added and the samples were dried by rotary evaporation. This step was repeated, followed by three washes with methanol. The samples were extracted with ethanol and washed six times with methanol-acetic acid and three times with methanol. Acetylation was performed with 1.0 ml of acetic anhydride and 10 μ l of pyridine at 80°C for 2 h. Toluene was added to the samples which were then dried by rotary evaporation. This step was repeated three times. The samples were then suspended in water and chloroform, with the acetates

recovered from the chloroform, and 1–3 μ l used for the analysis. Analysis was performed on a 3 ft $\times$ 2 mm ID glass column packed with SP-2340 3% on 100/120 Supelcoport (Supelco, Bellefonte, PA) with nitrogen as the carrier gas at a flow rate of 50 ml/min. The column temperature was programmed to run from 180°C to 240°C at 2°C/min with a 6 min initial hold. Reference standards of alditol acetate mixtures (Supelco) were used to determine relative retention times. Individual peaks were measured as the area under the peak, and the percentage of the total area represented by each peak was determined.

Analysis of fatty acids was performed by converting them to methyl esters as described by Metcalfe *et al.* (1966) and modified by Moss *et al.* (1974). Analysis was performed on a 10 ft $\times$ 2 mm ID $\times$ $\frac{1}{4}$ in OD glass column packed with SP-2100 DOH 3% on 100/120 Supelcoport (Supelco) with nitrogen as the carrier gas at a flow rate of 20 ml/min. The column temperature was programmed to increase from 150°C to 225°C at 4°C/min. Reference standards of methyl esters (Supelco) were used to determine relative retention times. Peaks were measured as described above.

Preparation of antisera

Antisera against whole bacterial cells and LPS vaccines were prepared in New Zealand white rabbits. All injections were intravenous. Antisera to whole cell H1B and J5 were prepared with formalin-killed cells (1×10^9) for the initial immunisation, followed by four, weekly booster immunisations with 1×10^9 live cells. Antisera to H1B-LPS and J5-LPS were prepared with LPS conjugated to methylated bovine serum albumin at a ratio of 1:1. Rabbits were immunised with five weekly increasing doses (5–250 μ g of carbohydrate). All rabbits were bled 5 days after the last injection.

Chick-embryo toxicity and neutralisation

Purified antigens were examined for toxicity in 10-day-old white leghorn chick embryos (Horsfall and Tamm, 1965). Graded doses of each antigen (100 μ l) were injected on to the chorio-allantoic membrane and the eggs were examined 24 and 48 h later for death of the embryo. The LD50 was determined by probit analysis. Neutralisation of toxicity by heat-inactivated antisera was measured by mixing a constant amount of antiserum with graded doses of antigen. The mixtures were incubated for 30 min at 37°C before inoculation into eggs. Eggs were examined as above and significant neutralisation was determined by the χ^2 test.

Serological techniques

Haemagglutination assays were performed according to the method of Neter *et al.* (1956). The titre recorded was the highest serum dilution that gave a 1+ agglutination pattern.

Enzyme-linked immunosorbent assays (ELISA) were

performed according to the method of Engvall and Perlmann (1972). Polyvinyl chloride microtitration plates (Dynatech, Alexandria, VA) were coated with 50 μ l of LPS diluted in carbonate buffer, pH 9.6, to a concentration of carbohydrate 50 μ g/ml. The plates were incubated at 37°C for 6 h and then overnight at 4°C. The test sera were diluted in PBS-Tween 20 (0.05% v/w), pH 7.4, and 50 μ l was added to each well. The plates were incubated at 37°C for 2.5 h and then washed with PBS-Tween 20. The enzyme conjugate, goat anti-rabbit IgG (Fc portion) or IgM horseradish peroxidase (Sigma), was diluted 1 in 1000 in PBS-Tween. After adding 50 μ l to each well, the plates were incubated for 1.5 h at 37°C and then washed with PBS-Tween 20. The substrate, 40 mg of *o*-phenylene-diamine (Sigma), was dissolved in 0.1 M citrate buffer, pH 5.0, and 50 μ l were added to each well. After incubating for 1 h at room temperature, the reactions were stopped by adding 25 μ l of 2.5 M H₂SO₄. The absorbance at 490 nm was determined with a Biotech Model ELISA reader and compared with values obtained with normal serum.

The two-dimensional immunoelectrophoretic (IEP) techniques used were those described by Axelsen *et al.* (1973), with 1% low endo-osmosis agarose (FMC, Rockland, MD).

Results

Biochemical analysis

Application of HIB-LPS or J5-LPS to the Sepharose C1-6B column resulted in the major carbohydrate peak eluting with the void volume, indicating a large molecule with a mol. wt of *c.* 2×10^6 . These fractions formed strong immunoprecipitation lines against specific rabbit LPS antisera in fused rocket immunoelectrophoresis and were pooled separately for the biochemical analysis. Because these pooled fractions were not lyophilised, percentages of protein and KDO were based on carbohydrate content instead of dry weight.

HIB-LPS. The Westphal extract of HIB-LPS contained no detectable DNA and 41 μ g of non-dialysable protein, which represented 5.9% of the amount of carbohydrate (table I); the amount of KDO, 18 μ g, was 2.5% of the carbohydrate content. The GLC analysis of HIB-LPS, with mannose as the internal standard, revealed the presence of galactose, glucose, glucoheptose and glucosamine in ratios of 2.4:2:1 (fig. 1). A peak at 19.04 min could not be identified by comparison with relative retention times of known hexoses or other available heptoses such as sedoheptulose or mannoheptulose. Different hydrolytic conditions did not alter the presence or size of this peak. Glucose was the most abundant hexose (36.8%), while galactose (18.3%) and glucoheptose (16.8%) were present in approximately equal amounts (table II). A small amount of

Table I. Biochemical composition of *H. influenzae* type b and *E. coli* J5 LPS extracts employed for GLC analysis

Extract	Carbohydrate (μ g)	KDO (μ g)	Protein (μ g)	DNA
HIB-LPS (Westphal)	694	18	41	N.D.
J5-LPS (Galanos)	252	188	23	N.D.

N.D. = not detected at a sensitivity of DNA 1.4 μ g/ml.

Table II. GLC analysis of *H. influenzae* type b LPS (Westphal) carbohydrates and fatty acids

Peak no.	Retention time (min)	Constituent	Area	Percentage of total area
<i>Carbohydrates</i>				
1	13.43	Mannose	419	N.C.
2	14.64	Galactose	318	18.3
3	16.48	Glucose	637	36.8
4	19.04	Unknown	338	19.5
5	23.05	Glucoheptose	292	16.8
6	35.16	Glucosamine	148	8.5
<i>Fatty acids</i>				
1	5.13	Laurate	1354	0.7
2	8.83	Myristate	151370	80.1
3	10.10	12-Methylmyristate	11694	6.2
4	11.06	3-Hydroxymyristate	14864	7.8
5	12.83	Palmitate	2725	1.4
6	16.52	Oleate	6857	3.6
7	17.17	Stearate	16224	N.C.

N.C. = not calculated.

glucosamine (8.5%), a core component, was also detected.

Specific fatty acids were identified by GLC analysis with stearate as the internal standard (fig. 1). Because no authentic standard was available, 3-hydroxymyristic acid was identified by its presence in *Salmonella minnesota* Re595 LPS (kindly supplied by Dr R. E. McCallum). Myristate was the most abundant fatty acid detected (80.1%), followed by 3-hydroxymyristate (7.8%), and 12-methyl myristate (6.2%) (table II). Peaks at 8.02, 9.67, and 19.36 min could not be identified by available fatty acid standards.

J5-LPS. The Galanos extract of J5-LPS was void of detectable DNA, but contained 23 μ g (9.1%) of non-dialysable protein (table I). Contrary to the findings with the HIB-LPS extracts, there was a considerable amount of KDO (188 μ g) compared with the total amount of carbohydrate (252 μ g). In

Fig. 1. GLC analysis of *H. influenzae* type b LPS (Westphal) carbohydrates (A) and fatty acids (B).

Table III. GLC analysis of *E. coli* J5-LPS (Galanos) carbohydrates and fatty acids

Peak no.	Retention time (min)	Constituent	Area	Percentage of total area
<i>Carbohydrates</i>				
1	13.50	Mannose	821	N.C.
2	16.57	Glucose	96	4.1
3	19.06	Unknown	1616	69.7
4	23.11	Glucoheptose	150	6.6
5	35.15	Glucosamine	455	19.6
<i>Fatty acids</i>				
1	5.12	Laurate	14046	39.4
2	8.65	Myristate	17910	50.2
3	11.21	3-Hydroxymyristate	2002	5.6
4	12.80	Palmitate	1713	4.8
5	17.17	Stearate	31772	N.C.

N.C. = not calculated.

the GLC carbohydrate analysis (fig. 2), small amounts of glucose (4.1%) and glucoheptose (6.6%) were recovered, while a large percentage of glucosamine was found (19.6%) (table III). Large amounts of the unknown peak no. 4 were present (69.7%).

In the GLC fatty-acid analysis (fig. 2), myristate (50.2%) and laurate (39.4%) were the predominant fatty acids, while small amounts of 3-hydroxymyristate (5.6%) and palmitate (4.8%) were also detected (table III). Several peaks were present that were unidentifiable (6.41, 7.98, 9.56, and 23.29 min).

Toxicity

J5-LPS was more toxic in chick embryos (LD₅₀ 100 ng of carbohydrate) than HIB-LPS (LD₅₀ 512 ng of carbohydrate) (table IV). A significant reduction in lethality of HIB-LPS ($p \leq 0.05$) occurred only when it was neutralised by HIB-LPS antiserum

Fig. 2. GLC analysis of *E. coli* J5-LPS carbohydrates (A) and fatty acids (B).

Table IV. Toxicity of *H. influenzae* type b and *E. coli* J5 LPS extracts in chick embryos

Dose (ng of carbohydrate)	Number of embryos killed/ number tested with	
	HIB-LPS*	J5-LPS†
10000	18/19	15/15
1000	40/45	14/15
100	24/50	27/40
10	0/10	5/20
1	0/10	0/10

* LD50 = 512 ng (determined by probit analysis).

† LD50 = 100 ng (determined by probit analysis).

(table V). Similarly, J5-LPS toxicity was neutralised only by J5-LPS antiserum.

Haemagglutination assay

The haemagglutination assay was used to examine cross-reactivity between HIB-LPS and J5-LPS (table VI). Both whole-cell HIB and HIB-LPS antisera had titres of 128 against HIB-LPS coated cells. However, a 4- to 8-fold decrease occurred when the antiserum was tested against J5-LPS-coated cells. Similar reductions in titre were seen when whole-cell J5 and J5-LPS antisera were examined.

Table V. Neutralisation of *H. influenzae* type b and *E. coli* J5-LPS chick-embryo toxicity with specific rabbit antisera

Antigen	Carbohydrate dose (μ g)	Number of embryos killed/number tested with			
		no serum added to LPS (controls)	normal rabbit serum added to LPS (controls)	antiserum against	
				HIB-LPS	J5-LPS
HIB-LPS	1	30/35	10/11	7/40*	14/25
HIB-LPS	0.1	13/20	10/13	0/10*	7/15
J5-LPS	10	5/5	...	...	1/11*
J5-LPS	1	14/15	13/15	25/30	8/30*
J5-LPS	0.1	6/10	1/6	6/15	0/10*

* P value < 0.05 as determined by χ -square analysis, when compared with controls that received LPS without serum.

Table VI. *H. influenzae* type b LPS and *E. coli* J-5 LPS cross-reactivity by hemagglutination assay

Rabbit serum tested	Antigen coating sheep RBCs	Titre
Anti-whole cell HIB	HIB-LPS J5-LPS	
Anti-HIB-LPS	HIB-LPS J5-LPS	128 32
Anti-whole cell <i>E. coli</i> J5	HIB-LPS J5-LPS	4 128
Anti-J5-LPS	HIB-LPS J5-LPS	4 32
Normal rabbit serum	HIB-LPS J5-LPS	N.D. N.D.

N.D. = not detected.

Table VII. IgG and IgM titres of rabbit antisera to specific antigens as determined by ELISA

Coating antigen	Titre of antiserum against				Titre of normal rabbit serum (control)	
	HIB-LPS		J5-LPS		IgG	IgM
	IgG	IgM	IgG	IgM		
HIB-LPS	625	625	< 5	5	< 5	< 5
J5-LPS	< 5	5	125	125	< 5	< 5

Elisa

ELISA was also used to test for cross-reactivity between HIB-LPS and J5-LPS (table VII). Minimal IgM cross-reactivity, and no IgG cross-reactions were detected.

Discussion

Two-dimensional immunoelectrophoresis

HIB-LPS and J5-LPS were examined by IEP with various rabbit antisera against HIB and J5 antigens to determine whether any differences existed between the LPS immunoprecipitation patterns of these extracts and to detect any cross-reactivity. The immunoprecipitation patterns of HIB-LPS and J5-LPS were similar (fig. 3). Both exhibited a peak above the antigen well that fused with a peak that migrated towards the anode. No cross-reactivity was detected with heterologous antisera but both anti-HIB-LPS and anti-J5-LPS sera reacted weakly with *S. minnesota* Re595 LPS (kindly supplied by Dr R. E. McCallum). Re595 LPS is a Re mutant containing only KDO and lipid A.

The purpose of this study was to examine the basis for J5-LPS cross-protection in HIB infection (Marks *et al.*, 1982b). HIB-LPS and *E. coli* J5-LPS extracts were, therefore, examined for biochemical content, toxicity, and serologic cross-reactivity.

In comparing HIB-LPS with J5-LPS biochemically, one obvious difference was the amount of KDO present. J5-LPS contained KDO 74% based on the carbohydrate content, while HIB-LPS contained 2.6%. In the GLC carbohydrate analysis, HIB-LPS and J5-LPS possessed similar carbohydrates, except for the absence of galactose in J5-LPS, but in different relative amounts. These carbo-

Fig. 3. Comparison of two-dimensional immunoelectrophoresis patterns of *H. influenzae* type b LPS and *E. coli* J5-LPS antisera: gel A—HIB-LPS vs. whole-cell HIB antiserum; gel B—HIB-LPS vs. HIB-LPS antiserum; gel C—J5-LPS vs. whole-cell J5 antiserum; gel D—J5-LPS vs. J5-LPS antiserum.

hydrates were glucose, glucoheptose, and glucosamine. In the lipid analyses, similar fatty acids were found but the percentages again were different. In J5-LPS, myristate and laurate were most abundant, while in HIB-LPS, myristate was the predominant fatty acid. Thus, the lipid-A region of HIB-LPS and J5-LPS both contain similar fatty acids. A Galanos extract of HIB-LPS was also analysed biochemically. No significant differences were found in any of the biochemical analyses (data not shown).

Toxicity of the LPS preparations determined in

chick embryos after initial attempts in CF1 mice indicated LD50 values above 50 mg/kg for HIB-LPS. This method was used because chick embryos are sensitive to extremely small quantities of endotoxin. HIB-LPS was approximately five times less toxic than J5-LPS. Raichvarg *et al.* (1981) reported that LPS from *H. influenzae* type a had an LD50 of 26.5 mg/kg in mice and the lipid-A fraction had an LD50 of more than 50 mg/kg. Thus, it seems that *H. influenzae* LPS may be generally less toxic than LPS from Enterobacteriaceae. The lipid-A region is

responsible for the toxicity of LPS (Galanos *et al.*, 1971) and the ester-linked fatty acids are known to be important in the degree of endotoxocity (Rietschel *et al.*, 1975). Even though HIB-LPS and J5-LPS have similar fatty acids, the quantity and arrangement may be important in determining certain biological activities.

The presence of common antigenic determinants between HIB-LPS and J5-LPS was examined by several different methods. HIB-LPS and J5-LPS antisera were unable to bind significantly (ELISA) or neutralise (chick embryo toxicity) heterologous LPS. There was no visible immunoprecipitation in IEP when HIB-LPS and J5-LPS antisera were tested against heterologous LPS. It is possible that J5-LPS, a rough LPS, may not precipitate as well as an LPS with an intact O antigen. This is made evident by an increase in solubility after conjugating J5-LPS with methylated BSA. In contrast, HIB antiserum was four- to eight-fold more potent than J5 antiserum in agglutinating heterologous LPS-coated sheep red-blood cells. This may indicate that J5-LPS induces an antibody that reacts with a site on HIB-LPS, such as a core component, that is less accessible. HIB-LPS may also induce small amounts of core antibody that react with J5-LPS. It is unlikely that this reaction is due to antibody against outer-membrane proteins, because when HIB-LPS was tested against a HIB outer-membrane-protein preparation in two-dimensional IEP, no immunoprecipitant lines were observed (not shown). Some evidence to implicate core antibody was demonstrated by a weak reaction of both HIB-LPS and J5-LPS antisera in two-dimensional IEP with the same immunoprecipitation pattern against *S. minnesota* Re595 LPS, which consists only of KDO and lipid A (not shown). Thus, the weak serological cross-reactivity seen with HIB-LPS and J5-LPS is probably due to a common immunodeterminant group in the core glycolipid. A recent report has shown that lipid A-specific monoclonal anti-

bodies to J5-LPS can be produced by immunising animals with whole heat-killed J5 cells. These antibodies cross-reacted with LPS from various gram-negative bacteria, including *S. minnesota* Re595 and clinical isolates of *Pseudomonas aeruginosa* (Mutharia *et al.*, 1984). Other anti-J5 monoclonal antibodies have been produced that cross-react with clinical isolates of *Klebsiella pneumoniae*, *Pseudomonas* sp. and *Proteus* sp. (Nelles and Niswander, 1984). Studies by Mullan *et al.* (1974) have shown that lipid A is not sufficiently exposed on the intact organism for antibody to be attached. Likewise, the antigenic determinants of lipid A and core glycolipid are distinct, presumably because of the masking effect of KDO (Johns *et al.*, 1977). However, since HIB-LPS has very little KDO, another core sugar, such as glucoheptose may be immunodominant, or antigenic determinants of the lipid region may be exposed. Flesher and Insel (1978) reported that HIB-LPS contained at least three O-antigenic determinants because of variations in the polysaccharide region. This was determined by haemagglutination and absorption experiments with LPS from all six capsular types (a-f). Our data indicate that there are common determinants in HIB-LPS and J5-LPS in the core region, although the reactivity is very weak. The results from these experiments do not explain the cross-protection previously reported between *E. coli* J5 and HIB infection in mice (Marks *et al.*, 1982b). However, other mechanisms besides biochemical or immunological similarities have not been explored. One such mechanism is enhanced bacterial clearance by opsonisation or a non-specific serum factor induced by immunisation with LPS (G. Dominique, personal communication).

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