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Protective activity of the antilipopolysaccharide antibodies from human cord serum

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Abstract

We evaluated the ability of human maternal and cord serum antibodies to protect mice challenged with live *Escherichia coli* serotype O6:K2ac (*E. coli* O6). Mice received paired maternal or cord serum pools before a challenge with *E. coli* O6 to evaluate the mortality rate. All the pools were able to protect the animals challenged with bacteria except the test group from paired maternal and cord sera from preterm neonates containing less than 1.0 mg L^{-1} immunoglobulin G antibody levels. In liver, spleen and mesenteric lymph nodes from the control group (phosphate–buffered saline), more than 10^2 CFU mL^{-1} bacteria were found at 30 min and more than 10^5 CFU mL^{-1} after 120 min. The test group showed lower bacterial counts in the organs, and no bacteria in the mesenteric lymph nodes during the evaluated period. Tumor necrosis factor alpha and interleukin 6 were undetectable in serum from animals pretreated with paired maternal and cord serum pools from full-term neonates and pools from preterm neonates containing high antibody and avidity levels. Our findings suggest that placental transfer of antilipopolysaccharide O6 immunoglobulin G antibodies to neonates has a high capacity to prevent lethal infection with *E. coli* O6 in a mouse protection model and that the degree of protection is determined by the concentration and avidity of these IgG antibodies.

Introduction

Gram-negative bacteria are prevalent in the maternal canal and can colonize infants before or during delivery (Klein *et al*., 2000). *Escherichia coli*, especially serotypes O4, O6, O7 and O16, are frequently isolated from blood samples of newborns with sepsis (McCabe *et al*., 1978; Cross *et al*., 1984; Kuseceket *et al*., 1984; Dias *et al*., 1994). The type of effect is determined by several factors including the virulence of the organisms, the size of the inoculum, and host nutritional status, age and prematurity (Hotchkiss & Karl, 2003). Despite advances in neonatal management, systemic bacterial infections remain an important cause of morbidity and mortality in infants, especially among low birthweight infants (Ng, 2004).

The interaction between lipopolysaccharide (LPS) and mononuclear cells does not injure host tissues directly but rather through the action of induced endogenous mediators of inflammation (Klein *et al*., 2000). Macrophages stimulated by LPS, in the presence of high levels of C5a, showed enhanced production of tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6), which could be related to cell damage (Ward, 2004). Increased susceptibility to sepsis in the neonates has been attributed to the lack of maternal anti-LPS antibodies, supposed to be only of the immunoglobulin M (IgM) isotype, according to current literature (Lewis & Wilson, 1995), which could partially explain the susceptibility of newborns to Gram-negative bacterial infections. Previous studies by our group demonstrated the existence of placental transfer of anti-LPS immunoglobulin G (IgG) antibodies, reactive not only to the O chain, but also against the core and lipid A (Nagao *et al*., 1998, 2001).

Chemically extracted LPS is frequently used in animal models to study human endotoxemia (Ge *et al*., 1994). It was demonstrated that the cellular localization differs when purified LPS compared with viable bacteria are injected (Ge *et al*., 1994). The findings raised the question of whether studies utilizing purified LPS preparations to verify biological activities in animal models could truly reproduce the biological properties of LPS released from live bacteria (Ge *et al*., 1994).

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Selective breeding of mice from outbred stock was carried out for quantitative antibody response to flagellar and somatic antigens of salmonellae (Mice Selection III) (Siqueira *et al.*, 1976). At the selection limit, this selection produced homozygous high (HIII) and low (LIII) responder lines for the character investigated – the peak agglutinin response to optimal secondary immunization (Sant'anna *et al.*, 1989). This phenotype was shown to be controlled by approximately seven independent loci expressing themselves at several distinct cellular levels (Sant'anna *et al.*, 1989). Several quantitative trait loci, which regulate antibody production against many other antigens, were demonstrated recently. These two lines of mice have shown strong biologically significant differences, as the interline difference in antibody levels was not restricted to the antigen used for the selection (multispecific effect) and were associated with modifications in resistance to infections and experimental arthritis, longevity and tumor incidence (Sant'anna *et al.*, 1989; Souza *et al.*, 2004).

Considering a putative role of anti-LPS antibodies as the basis for immunoprophylaxis or immunotherapy for Gram-negative sepsis (Pollack, 1999), we speculated whether the cord serum antibody repertoire, which obviously represents maternal IgG, would be able by itself to protect inbred HIII mice from a challenge with live *E. coli* O6.

Materials and methods

Serum samples

Blood samples were collected from healthy, well-nourished mothers aged 18–35 years and cord blood samples from their respective newborn infants, after informed consent. All mothers and infants were selected from among those cared for at the Hospital Maternidade Cachoeirinha, São Paulo, Brazil. Most of the mothers lived under poor socioeconomic and hygiene conditions. Twenty-four newborn infants were full-term and adequate for gestational age and 12 were preterm infants under 37 weeks of gestational age. After blood collection, serum samples were obtained and kept at –20 °C until analysis. The project was approved by the Ethics Committee for Human Research (ECHR), Instituto de Ciências Biomédicas/Universidade de São Paulo – on 06/16/1998.

Bacteria

Escherichia coli (serotype O6 : K2ac : H1) was provided by the Center of Culture Collection, Gothenburg, Sweden.

LPS extraction

LPS was extracted with hot phenol by the method of Westphal & Jann (1965). Briefly, a 20 L culture of *E. coli* O6 : K2ac : H1 was obtained and submitted to centrifugation at 10 000 ***g*** for 30 min (Sorvall superspeed RC2-B, Reston, VA). The pellet was washed twice in acetone and frozen at − 70 °C. The bacterial paste (29 g) was dissolved in distilled water, warmed to 67 °C and mixed (v/v) with prewarmed phenol (Merck, Germany). The mixture was shaken vigorously for 15 min, cooled and centrifuged at 18 000 ***g*** for 15 min. The aqueous phase was recovered and re-extracted twice more with phenol. The final suspension was extensively dialyzed against distilled water and LPS was precipitated by the addition of ethyl alcohol to a final concentration of 80%. The precipitate was recovered by centrifugation at 18 000 ***g*** for 15 min. After re-suspension in distilled water, the solution was ultracentrifuged at 100 000 ***g*** for 4 h and the final pellet was lyophilized and tested for protein and nucleic acid content. This preparation contained less than 1% nucleic acid and 2.1% proteins.

Animals

Inbred male HIII (high III) mice, six to eight weeks old (kindly donated by Dr Maria Siqueira, Butantan Institute, São Paulo, Brazil), selected for high susceptibility to infection with *Salmonella typhimurium* (Siqueira *et al.*, 1976; Sant'anna *et al.*, 1989; Souza *et al.*, 2004) were used. The HIII selection of mice began in 1976, done by Dr Maria Siqueira as an outbred selection for high susceptibility to *S. typhimurium* infection (Sant'anna *et al.*, 1989). In 1997, the inbreeding process began, and after 14 generations, the HIII mice were transferred to an isolator, where the inbreeding process continued. Today, it is in the F36 generation of inbreeding (Cabrera, WHK, Instituto Butantan, São Paulo, Brazil, pers. comm.).

The project was approved by the Ethics Committee for Animal Research (ECAR), Instituto de Ciências Biomédicas/Universidade de São Paulo.

Enzyme-linked immunosorbent assay (ELISA) for antibody levels

Microtiter plates (Costar) were coated with 10 mg L^{-1} of purified LPS O6 diluted in phosphate-buffered saline (PBS), 100 μL per well. After blocking the plates with 5% fish gelatin (Sigma, St Louis, MO), the reference standards for serum IgG, IgM and immunoglobulin A (IgA) antibody isotypes, paired maternal and cord serum samples diluted to 1 : 50 in PBS-0.5 M NaCl-0.2% Tween 20, were incubated in duplicate in the wells at 4 °C overnight. After washing with PBS-0.5 M NaCl-0.2% Tween 20, plates were incubated with horseradish peroxidase-conjugated goat anti-human IgG, IgM or IgA (Sigma) for 2 h at 37 °C. The substrate solution containing 0.4 mg orthophenylenediamine per mL citrate-phosphate buffer 0.1 M at pH 5.0 was added to the plates. After 30 min incubation, the enzymatic reaction was stopped with 50 μL of 2.5 N H_2SO_4. Absorbances were read

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in a microplate reader at 492 nm (Labsystems Multiskan MS, Farnborough, Hampshire, UK). A pool of blood donor serum samples, included in the assays for anti-LPS IgG, IgM and IgA antibody measurements, were used as second reference standards. The concentrations of the standards were determined according to the procedure reported by Fomsgaard (1990). Purified human IgG and IgA (codes I-8640 and I-2636, Sigma) and a commercial human serum sample for IgM values (catalog 552, Kallestad, MI) were used as primary reference standards. After analysis, the sera were pooled according to the total maternal antibody levels. The concentrations of anti-LPS O6 IgG, IgM and IgA antibodies were then determined in the pools from paired maternal and cord serum.

ELISA for antibody avidities

The serum antibody avidities of the pools from paired maternal and cord serum were determined using potassium thiocyanate to elute bound complexes (Jones *et al.*, 1987). Microtiter plates (Costar) were coated with $10\,mg\,L^{-1}$ of purified LPS O6 diluted in phosphate buffered saline (PBS), 100 μL per well. After blocking the plates with 5% fish gelatin (Sigma), the pooled serum samples were diluted to 1 : 50 in PBS-0.5 M NaCl-0.2% Tween 20 (in duplicates), and incubated overnight at 4 °C. After incubation and washing, 0.1 mL potassium thiocyanate (0.0, 0.10, 0.25, 0.5, 1.0, 2.0, 3.0 and 4.0 M) in PBS-0.5 M NaCl-0.2% Tween 20 were added to the wells. After 30 min, the plates were washed six times and incubated for 2 h with horseradish peroxidase-conjugated goat anti-human IgG, IgA or IgM (Sigma). The plates were washed three times and incubated with 0.1 mL of the substrate solution. The results were expressed as the molarity of potassium thiocyanate necessary to elute 50% of the bound complexes.

Animal model

The lethal dose 100 (LD_{100}) of the bacteria was determined by challenging adult HIII mice with viable *E. coli* O6 (1×10^6 to 7×10^6 CFU per animal) intraperitoneally. The number of dead animals was assessed at regular intervals over a period of 72 h.

Protective experiments with maternal and cord serum pools from full-term or preterm neonates

Adult HIII mice were injected intraperitoneally with 0.4 mL of paired maternal or cord serum pools from full-term or preterm neonates containing high or low levels of anti-LPS O6 antibodies (test groups) or with PBS (control groups) 18 h before being challenged intraperitoneally with 3×10^6 viable *E. coli* O6 CFU per animal. Survival of the animals was assessed at regular intervals over a period of 72 h. For organ extraction, some animals were sacrificed 24 h after the challenge and the organs were submitted to microbiological and histochemical analysis.

Microbiological analysis

Brain, spleen, heart, liver and mesenteric lymph nodes from the control and test groups were removed by using a sterile aseptic technique, weighed, and homogenized in 8 mL of Brain Heart Infusion (BHI, Difco, Detroit, MI). A 10 μL volume of a 10^{-4} dilution of each extract was cultivated onto BHI agar (Difco) plates. After 18 h incubation at 37 °C, the number of bacteria was estimated.

Histochemical analysis

Livers from the control and test groups were left in 5% formaldehyde solution for 24 h and then embedded in 70% ethanol and paraffin. The blocks were cut into 3–5 μm sections and transferred to microscope slides. In addition to the routine hematoxylin and eosin staining, the sections were stained for Ziehl-Neelsen stain to identify *E. coli* O6. The sections were analyzed at 1000 × magnification under a light (Zeiss, model LSM 510; Jena, Thuringia, Germany).

Prevention of bacterial translocation

To evaluate the capacity of cord serum antibodies to prevent bacterial translocation, HIII mice were injected intraperitoneally with 0.4 mL full-term cord serum pool containing high levels of anti-LPS O6 antibodies 18 h before being challenged with the live bacteria. Groups of four animals were sacrificed every 30 min (30, 60, 90, 120 min) after the challenge. Spleen, liver and mesenteric lymph nodes were homogenized with 1 mL of sterile PBS. A 10 μL volume of a 10^{-4} dilution of each extract was cultivated on BHI agar plates. After 18 h incubation at 37 °C, the colonies were counted. Blood was collected from the animals by retro-orbital puncture. Serum samples were tested for the presence of circulating human antibodies by ELISA.

IL-6 and TNF-α measurement in mouse serum

Adult mice (four per group) were injected intraperitoneally with 0.4 mL paired maternal or cord serum samples from full-term and preterm neonates, 18 h before being challenged with 10 μg of LPS O6 per animal, intraperitoneally. At 0, 2 and 6 h after the challenge, the mice were sacrificed and blood was collected by retro-orbital puncture. Serum levels of IL-6 and TNF-α were measured using ELISA (Pharmingen, San Diego, CA).

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ELISA for cytokines

Microtiter plates (Costar) were coated with antimouse IL-6 or TNF-α antibodies (Pharmingen), diluted in 0.1 M carbonate buffer, pH 9.5, and incubated overnight at 4 °C. After washing with PBS-0.05% Tween, the plates were blocked with PBS containing 10% fetal bovine serum for 1 h at room temperature. After washing, serum samples from the animals and the reference standards were diluted with PBS-10% fetal bovine serum, and incubated in duplicate overnight at 4 °C. Samples were assayed at fourfold serial dilutions starting at a dilution of 1 : 2. After washing with PBS-0.05% Tween, the plates were incubated with 1 : 250 biotinylated antimouse IL-6 and antimouse TNF-α conjugates and the streptavidin-horseradish peroxidase conjugate for 1 h at room temperature. After washing the plates, the substrate solution containing 0.4 mg orthophenylenediamine mL^{-1} and 0.01% hydrogen peroxide in 0.1 M citrate-phosphate buffer, pH 5.0, was added. After 30 min incubation, the enzymatic reaction was stopped by adding 50 μL of 2.5 M H_2SO_4. Absorbances were read in a microplate reader at 450 nm (Labsystem Multiskan MS, USA). The results were determined using the GraphPad Prism 1.2 (San Diego, CA) program and expressed in mg L^{-1}.

Statistical analysis

The non-parametric Wilcoxon test, Friedman test and Spearman's correlation were used for statistical analyses of maternal and cord serum antibodies and serum pools. Data concerning the mortality rates for animals grouped by anti-LPS O6 IgG antibody concentrations were analyzed statistically by the Chi-squared test. IL-6 and TNF-α levels were compared among groups by the Tukey test. The level of significance was set at $P < 0.05$ in all analyses.

Results

Levels of anti-LPS O6 antibodies

Anti-LPS O6 antibody levels in maternal and cord serum samples are shown in Table 1. Anti-LPS O6 IgG antibody levels in cord serum from full-term newborns were similar to their respective maternal IgG antibody levels (Wilcoxon test, $P > 0.1$), and a significant correlation was found between levels of IgG antibodies in mothers and neonates (Spearman's correlation, $r = 0.85$, $P < 0.0001$). Similar data were obtained for preterm neonates and their respective mothers (Wilcoxon test, $P > 0.1$, and Spearman's correlation, $r = 0.81$, $P = 0.0004$). Comparing concentrations of different isotypes in maternal sera, anti-LPS O6 IgM levels were significantly higher than the other antibody isotypes (Friedman's test, $P < 0.0001$, Table 1). Specific IgA levels were not significantly different from the IgG antibody levels.

The formation of the pools was necessary because the volume of the samples from maternal and cord was low and each experiment utilized 0.4 mL per animal. Antibody isotype levels were measured by ELISA and then grouped according to the total maternal antibody levels. For the formation of the pools, high antibody levels were hypothetically considered to be above 20 mg L^{-1}. The only isotype present in cord serum was IgG and therefore cord serum samples were paired with maternal samples. Anti-LPS O6 IgG antibody levels in the pools of cord serum from full-term and preterm neonates were similar to those of the respective mothers and showed a strong correlation with the maternal levels (Spearman test, $P < 0.0001$, $r = 0.95$, Table 2). The avidity indexes of anti-LPS O6 in the pools of cord serum from full-term and preterm neonates (Table 3) did not differ from maternal antibody avidities (Wilcoxon test, $P = 0.45$ and $P = 0.30$, respectively).

Animal model

For the animal model, HIII male mice were challenged with 1×10^6 ($n = 5$), 1.4×10^6 ($n = 8$), 3×10^6 ($n = 8$) and 7×10^6 ($n = 8$) CFU per animal of viable *E. coli* O6. All mice injected with 3×10^6 and 7×10^6 CFU per animal of *E. coli* O6 died between 18 and 24 h. The lethal dose 50 (LD_{50}) was between 1×10^6 and 1.4×10^6, and the lethal dose 100 (LD_{100}) could be reached with 3×10^6 CFU per animal. Therefore, we

Table 1. Anti-LPS O6 antibody concentrations (mg L^{-1}) in maternal and cord sera from their respective full-term and preterm neonates

	Maternal serum			Cord antibodies*
	IgA	IgM	IgG	IgG
Full-term neonates				
$n^\dagger$	24	24	24	24
Mean	1.6	17.4	1.6	1.5
SEM	0.1	2.9	0.5	0.5
Lower 95% CI	1.4	11.3	0.4	0.5
Upper 95% CI	1.9	23.5	2.7	2.5
Friedman test (*P*)		<0.0001		<0.0001
Preterm neonates‡				
$n^\S$	12	12	12	13
Mean	93.2	13.3	1.1	1.0
SEM	24.6	4.3	0.2	0.2
Lower 95% CI	36.5	3.8	0.5	0.5
Upper 95% CI	149.9	22.8	1.3	1.5
Friedman test (*p*)		<0.0001		<0.0001

*The only isotype present in cord serum was IgG.

†Serum samples from full-term newborn infants, adequate for gestational age and their respective mothers.

‡The results of samples 12 and 13 belong to the same mother that gave birth to gemellary babies.

§Serum samples from preterm newborn infants under 37 weeks of gestational age and their respective mothers.

CI, confidence interval.

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Table 2. Levels of anti-LPS O6 antibodies in the pools of paired maternal and cord serum from full-term and preterm neonates containing high and low concentrations (mg L^{-1}) of antibodies. The serum samples were grouped according to the maternal antibody levels

	Maternal serum (mg L^{-1})*			Cord serum (mg L^{-1})†
	IgA	IgM	IgG	IgG
Full-term neonates				
n‡	24	24	24	24
High levels§	1.5	25.0	1.4	1.3
Low levels†	0.8	13.2	1.1	0.9
Preterm neonates				
n¶	12	12	12	13
High levels‖	2.1	18.0	9.7	9.7
Low levels§	0.8	12.2	0.9	0.8

*The levels of anti-LPS O6 antibody isotypes were determined by ELISA and grouped according to the maternal antibody levels.
†The only isotype present in cord serum was IgG.
‡Twenty-four newborn infants were full-term and adequate for gestational age.
§Low levels of anti-LPS O6 antibodies were hypothetically considered to be below 20 mg L^{-1} and cord serum samples were paired with maternal samples.
¶Thirteen newborn infants were preterm infants under 37 weeks of gestational age.
‖High levels of anti-LPS O6 antibodies were hypothetically considered to be above 20 mg L^{-1} and cord serum samples were paired with maternal samples.

Table 3. Anti-LPS O6 antibody avidities in pools of paired maternal and cord serum from full-term and preterm neonates containing high and low concentrations. The relative avidity indexes was expressed as potassium thiocyanate molarities (M) necessary to elute 50% of the bound complexes (antigen-specific antibodies) in ELISA

	Maternal serum (m)*			Cord serum (m)†
	IgA	IgM	IgG	IgG
Full-term neonates				
n‡	24	24	24	24
High levels§	>4.0	2.8	<4.0	>4.0
Low levels¶	>4.0	2.6	3.9	3.6
Preterm neonates				
n‖	12	12	12	13
High levels§	3.2	2.7	>4.0	>4.0
Low levels¶	>4.0	2.8	2.7	3.3

*The avidity indexes of the pooled antibodies were determined by ELISA and cord serum samples were paired with maternal samples.
†The only isotype present in cord serum was IgG.
‡Twenty-four newborn infants were full-term and adequate for gestational age.
§High levels of anti-LPS O6 antibodies were hypothetically considered to be above 20 mg L^{-1}.
¶Low levels of anti-LPS O6 antibodies were hypothetically considered to be below 20 mg L^{-1}.
‖Thirteen newborn infants were preterm infants under 37 weeks of gestational age.

established 3×10^6 CFU of viable bacteria per mouse as the necessary dose to obtain a death rate of 100%.

Protective experiments with maternal and cord serum pools from full-term or preterm neonates

All pools were able to protect the animals challenged intraperitoneally with viable bacteria, except the maternal- and cord serum pools from preterm neonates containing IgG antibody levels below 0.9 mg L^{-1}, and the control group (PBS), as shown in Figs 1 and 2. The microbiological analysis of the spleen, brain, heart, liver, mesenteric lymph nodes, lung and kidney from the animals revealed high numbers of bacteria ($>10^{10}$ CFU) in the control group, but a low number of bacteria ($\leq 10^3$ CFU) in the group which had received cord serum from full-term neonates with high antibody levels (Table 4). Histochemical analysis showed no stained bacteria inside the hepatocytes of the animals pretreated with serum from full-term and preterm neonates containing high antibody levels, in contrast to the control group, which showed hepatocytes completely invaded by the bacteria (Fig. 3).

Prevention of bacterial translocation

The prevention of the dissemination of *E. coli* O6 from the peritoneal cavity to sterile sites was evaluated at different times after inoculation of the bacteria in the animals. Liver, spleen and mesenteric lymph nodes from the control group presented more than 10^2 bacterial CFU mL^{-1} at 30 min and more than 10^5 CFU mL^{-1} after 120 min. The test group, which had received cord serum from full-term neonates with high antibody levels, showed significantly lower bacterial counts (below 10^3 CFU mL^{-1}) in the organs, and no bacteria in mesenteric lymph nodes during the period evaluated (Fig. 4). To confirm the presence of the human antibodies, mouse serum was collected at different times and checked by ELISA. The serum sample presented 118.0 μg L^{-1} at 30 min, 125.0 μg L^{-1} at 60 min, 113.0 μg L^{-1} at 90 min and 130.0 μg L^{-1} at 120 min, of human anti-LPS O6 IgG antibodies. The samples showed a mean concentration of 121.5 μg L^{-1} human IgG antibodies. No decay of the passively transferred antibodies was observed during the time analyzed.

Detection of IL-6 and TNF-α in animal serum

IL-6 and TNF-α levels were measured by ELISA in sera from animals challenged intraperitoneally with 10 μg of LPS O6 per mouse. As shown in Table 5, the cytokine levels in serum from the animals treated with PBS did not differ significantly from levels in serum from the animals pretreated with paired maternal and cord sera from preterm neonates containing low antibody levels ($P > 0.05$). On the other

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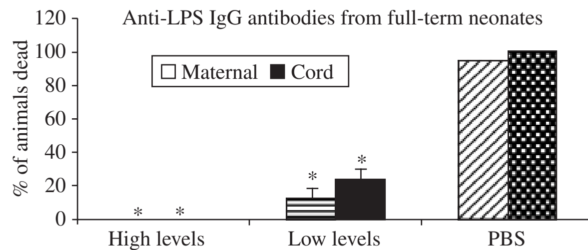

Fig. 1. Evaluation of the functional activity of human antibodies in protecting animals from challenge with 3×10^6 CFU of viable *Escherichia coli* O6 per mouse. The tested were paired maternal and cord serum pool from full-term neonates containing high and low anti-LPS O6 IgG antibody levels. Data concerning the mortality rates for animals grouped by anti-LPS O6 IgG antibody concentrations were statistically analyzed by the Chi-squared test. Results represent the standard deviation of 17 animals per group. Significant differences were considered at levels of $P < 0.05$ in maternal and cord serum groups.

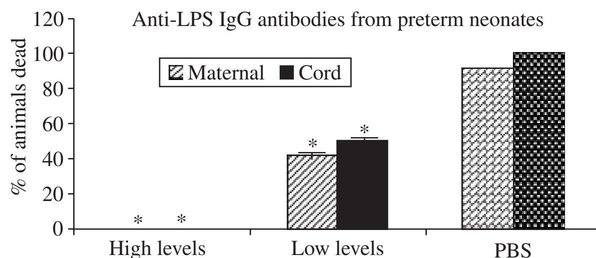

Fig. 2. Evaluation of the functional activity of human antibodies in protecting animals from challenge with 3×10^6 CFU of viable *Escherichia coli* O6 per mouse. The tested samples were paired maternal and cord serum pools from preterm neonates containing high and low anti-LPS O6 IgG antibody levels. Data concerning the mortality rates for animals grouped by anti-LPS O6 IgG antibody concentrations were statistically analyzed by the Chi-squared test. Results represent the standard deviation of 12 animals per group. Significant differences were considered at levels of $P < 0.05$ in maternal and cord serum groups.

hand, the cytokines were undetectable in serum from animals pretreated with paired maternal and cord serum from full-term neonates and in serum from animals pretreated with paired maternal and cord serum from preterm neonates containing high antibody levels ($P < 0.001$).

Discussion

A high prevalence of sepsis caused by enterobacteria such as *E. coli* is observed during the neonatal period (Siegel & McCracken, 1981). In a study conducted in São Paulo, Brazil, it was found that 6.4% of sepsis was caused by *E. coli* (Brandileone *et al.*, 1984). In another study, it was shown that *E. coli* O6 serotype was frequently isolated in samples from newborns with sepsis (Dias *et al.*, 1994).

Based on our previous data showing that anti-LPS O6 antibodies in cord blood were similar to those found in the respective maternal serum samples (Nagao *et al.*, 1998), we questioned whether these antibodies would be able to protect mice from the challenge with live *E. coli* O6 strain.

Due to the small quantity of our serum samples for the evaluation of protective capacity of the antibodies anti-LPS O6 in mice challenged with live bacteria, these samples were pooled according to the maternal antibody levels.

In the protective experiments, we demonstrated that all mice that received the pool of cord serum from full-term neonates containing $1.3\,\mathrm{mg\,L^{-1}}$ of IgG antibodies and avidity indexes above 4 M or the pool of cord serum from preterm newborns containing $9.7\,\mathrm{mg\,L^{-1}}$ of IgG antibodies and avidity indexes above 4 M survived. The pool of cord serum from full-term neonates containing $0.9\,\mathrm{mg\,L^{-1}}$ IgG antibodies and avidity indexes of 3.6 M protected 76% of the animals, and the pool of cord serum from preterm newborns containing $0.8\,\mathrm{mg\,L^{-1}}$ and IgG antibodies avidity indexes of 3.3 M was able to protect only 50% of the animals. This suggests that the differences in survival rates did not depend on the IgM antibody concentrations, but on the IgG antibody levels. These results agree with our recent work (Pontes *et al.*, 2005) showing that purified IgG containing at least $1.0\,\mathrm{mg\,L^{-1}}$ of anti-LPS O6 IgG antibodies was able to protect mice from the challenge with viable *E. coli* O6. Kim *et al.* (1988) demonstrated that lower concentrations of anti-LPS O18 IgG antibodies (15- to 100-fold less than IgM) presented a high protection capacity in rats challenged with O18:K1 *E. coli*.

Table 4. Bacterial translocation in culture of organ extracts from male HIII mice, injected with 0.4 mL of cord serum pool from full-term neonates (Test group) or phosphate-buffered saline (Control group), 18 h before the challenge with viable *Escherichia coli* O6. For the evaluation of the bacterial translocation, the animals were sacrificed 24 h after the challenge and the organs were homogenized, diluted to 10^{-4} in BHI broth and soon after, each extract was cultivated onto BHI agar plates for 18 h at 37 °C. After incubation, the number of bacteria was evaluated as CFU per organ

Organ*	Control group†	Test group‡
Spleen	3.6×10^{10}	1×10^3
Brain	3.4×10^{10}	2×10^1
Heart	3.2×10^{10}	0
Liver	3.4×10^{10}	1×10^3
Mesenteric lymph nodes	3.4×10^{10}	0
Lung	3.0×10^{10}	1×10^3
Kidney	3.2×10^{10}	0

*Bacterial culture of organ extracts from eight animals per group challenged with viable 3×10^6 CFU per mouse of *E. coli* O6.
†A 10 μL volume of 10^{-4} dilution of each extract was cultivated on BHI agar plates for 18 h at 37 °C, and the number of bacteria was evaluated as CFU per organ.
‡Cord serum pool from full-term neonates containing high levels of anti-LPS O6 antibodies.

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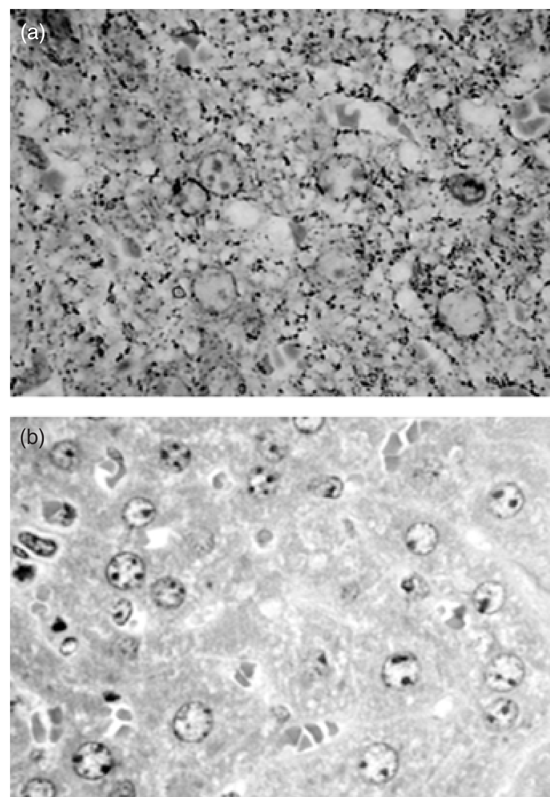

Fig. 3. Histochemical analysis of the livers of mice challenged with 3×10^6 CFU per animal of *Escherichia coli* O6, intraperitoneally 18 h after having received cord serum pool from full-term neonates containing high levels of antibodies. Photomicrography shows a high number only in the control group. (a) Control group (mice injected with PBS) – not protected animals. (b) Test group (mice pretreated with cord serum from full-term neonates containing high levels of anti-LPS O6 IgG antibody) animals with previous injection of cord serum from full-term neonates containing high levels of anti-LPS O6 IgG antibody. In addition to the routine hematoxylin and eosin staining, the microscope slides were stained for Ziehl-Neelsen stain to identify *E. coli* O6. The sections were analyzed at 1000 × magnification under a light.

Sepsis in neonates can originate from the phenomenon of virulent bacterial strains translocated from the gastrointestinal tract, or from other sites, to the bloodstream under certain circumstances, reaching organs such as the liver and the spleen (Schiff *et al*., 1993; Stuber, 1999; Ljungdahl *et al*., 2000; Naaber *et al*., 2000). To check the translocation of *E. coli* O6 : K2ac, the bacterial counts in the organ extracts from animals pretreated with cord serum pool from full-term neonates containing 1.3 mg L^{-1} antibodies were analyzed: absent or low (below 10^4) numbers of CFU were found. In contrast, analysis of the bacterial counts of the control group showed high numbers of CFU, above 10^{10} in all the analyzed organs. Our next step was to study the progression of bacterial dissemination to the organs by sacrificing a number of test and control animals every 30 min after the bacterial challenge. Within the first 30 min,

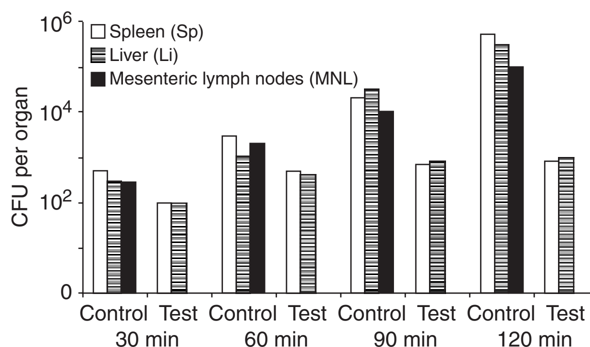

Fig. 4. Analysis of bacterial translocation of the spleen, liver and mesenteric lymph nodes from HIII mice challenged with 3×10^6 CFU per mouse of viable *Escherichia coli* O6 intraperitoneally 18 h after having received cord serum pool from full-term neonates containing high levels of anti-LPS O6 IgG antibodies. For the analysis, groups of four animals were sacrificed every 30 min (30, 60, 90 and 120 min) after the challenge. A 10 μL volume of a 10^{-4} dilution of each extract was cultivated on BHI agar plates. After 18 h incubation at 37 °C, the number of bacteria was evaluated as CFU per organ.

Table 5. Serum concentrations (μg L^{-1}) of TNF-α and IL-6 of animals injected with maternal serum and cord serum pools from full-term and preterm neonates containing high or low levels of anti-LPS O6 antibodies or PBS, 18 h before being challenged with 10 μg of LPS O6 per mouse. Results represent the SE of four animals per group. Significant differences were considered at levels of $*P < 0.001$ in maternal serum and cord serum pools from full-term neonates containing high or low levels of anti-LPS O6 antibodies and preterm neonates containing high levels of anti-LPS O6 antibodies

Groups†	n‡	TNF-α§,¶	IL-6§,¶
PBS	4	15.4 ± 2.3	22.8 ± 1.2
NPT low	4	7.2 ± 0.2	6.8 ± 0.5
MPT low	4	7.7 ± 0.2	7.8 ± 0.4
NPT high	4	0.0*	0.0*
MPT high	4	0.0*	0.0*
NT low	4	0.0*	0.0*
MT low	4	0.0*	0.0*
NT high	4	0.0*	0.0*
MT high	4	0.0*	0.0*

$*P < 0.001$.

†M, maternal serum pool; N, cord serum pool; T, full-term; PT, preterm pool; low, low levels of anti-LPS O6 antibodies, high, high levels of anti-LPS O6 antibodies.

‡n, number of animals tested per group.

§At 2 h (TNF-α) and 6 h (IL-6) after the challenge with LPS O6, the mice were sacrificed and blood was collected by retro-orbital puncture. Serum levels of IL-6 and TNF-α were measured by ELISA.

¶TNF-α and IL-6 levels were compared among groups by the Tukey test. The results are expressed as mean ± SEM of cytokine levels.

bacterial translocation to liver and spleen occurred in both the test and control animals, whereas translocation to mesenteric lymph nodes occurred only in the control group. During the subsequent minutes, the rate of dissemination

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remained the same for the test group, i.e. around 10^2 in liver and spleen, with no counts in mesenteric lymph nodes. However, in the control group, the bacterial counts increased logarithmically to more than 10^5 in the organs. Similar results have been reported by Naaber *et al*. (2000). These authors demonstrated that *E. coli* translocation from the peritoneal cavity to heart blood, mesenteric lymph nodes, liver, spleen, lung, heart muscle and kidney occurred within 45 min after inoculation, reaching the highest rate between 2 and 24 h after challenge with the bacteria (Naaber *et al*., 2000).

The injection of LPS or bacteria (*E. coli* O55:B5) in animal models or healthy volunteers results in excessive production of proinflammatory cytokines. It is believed that in the course of septic shock there is an excessive activation of the inflammatory cascade resulting in endothelial injury, organ injury and cardiovascular collapse (Kirsch & Giroir, 2000). This inflammatory cascade occurs as a result of high concentrations of C5a, enhancing the production of cytokines (Schiff *et al*., 1993; Stuber, 1999; Ward, 2004). Taking TNF-α and IL-6 as indicators of endotoxin shocks, we observed that animals that had received serum pool from full-term or preterm neonates containing 0.9 mg L^{-1} and 0.8 mg L^{-1} of anti-LPS O6 IgG antibodies, respectively, or from their respective mothers before the challenge with LPS O6, presented high levels of TNF-α and IL-6, similarly to the control animals. In contrast, the animals protected with cord serum from full-term neonates containing 1.3 mg L^{-1} of anti-LPS O6 IgG antibodies or preterm neonates with 9.7 mg L^{-1} of anti-LPS O6 IgG antibodies did not show any detectable proinflammatory cytokine in serum. These data agree with another recent study which demonstrated that animals protected with purified IgG from a pool of normal human serum containing 1.1 mg L^{-1} of anti-LPS O6 IgG antibodies showed no detectable cytokines (Pontes *et al*., 2005).

The mothers included in the present study are at a low socioeconomic level and live in houses with an adequate water supply but an irregular sewage system, a factor that increases the environmental exposure to microbes. The immune response can be adapted to a large number of environmental antigens by producing a greater amount of antibodies than in the presence of low environmental antigenic exposure (Nagao *et al*., 1993, 1995), even with possible lower avidity (Roberton *et al*., 1988). According to Zinkernagel (2001), high hygiene standards have reduced the induction and maintenance of protective maternal antibodies before pregnancy, which may consequently affect maternally acquired immune protection.

In conclusion, we demonstrated that full-term and preterm neonate serum samples and their respective maternal serum samples have a high capacity to protect animals from death, depending on the concentration and avidity of anti-LPS IgG antibodies. The model proposed in the present study may be adequate for further investigations of the use of gammaglobulins in sepsis. For this purpose, we suggest selecting serum samples from healthy people containing IgG antibody levels above 1.0 mg L^{-1} and an avidity index above 4 M for the production of an appropriate intravenous immunoglobulin preparation.

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