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Antibodies to O-Antigen of Lipopolysaccharide are Protective against Neonatal Infection with *Escherichia coli* K1

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Monoclonal IgM specific for the O18 antigen conferred passive protection to 1-week-old rats against bacteremia and killing after oral challenge with O18:K1 *Escherichia coli*. Specific protection of the pups was also achieved by immunizing the pregnant rats with purified O18 lipopolysaccharide. We suppose that most human newborns that are colonized by potentially invasive K1 *E. coli* are protected by the transplacental transfer of anti-lipopolysaccharide immunoglobulin G, and we suggest that treatment with such antibodies might in the future be considered a therapeutic option. Rat serum from 1-week-old animals had only about one-third of the complement hemolytic activity of adult rat serum. This low level of hemolytic activity correlated with a relatively poor bactericidal activity in antibody-dependent and antibody-independent bactericidal in vitro assays. Monoclonal anti-O18 immunoglobulin M, although protective in vivo and bactericidal when added to adult rat serum, only poorly inhibited the multiplication of O18:K1 cells in serum from 1-week-old rats. This suggests that other elements of host defense besides complement participate in antibody-mediated in vivo protection.

Escherichia coli strains of the K1 capsular serotype represent about 80% of all *E. coli* bacteria isolated from cases of meningitis in newborns (25, 26, 29). Most of the K1 isolates belong to a limited spectrum of O-lipopolysaccharide (LPS) antigen types. Data have been presented elsewhere showing that pyelonephritis and meningitis in newborns can be differentially associated with K1 bacteria of different O serotypes (14, 24). Here we concentrate on their virulence with regard to newborn meningitis. O1:K1, O7:K1, and O18:K1 bacteria have all been isolated from fecal samples of healthy individuals and from newborns with meningitis. However, the relative frequency of isolation of these bacteria differs almost eightfold between these two sources, and O1:K1 bacteria were therefore concluded to be relatively avirulent with regard to neonatal meningitis compared to the two other bacterial groups (24).

Distinct differences were observed when these groups of bacteria were tested for the ability to cause bacteremia after colonizing the gut of 5- to 7-day-old rats (24). All of these K1 strains colonized the gut and translocated to the mesenteric lymph nodes with comparable efficiency. The O7:K1 and O18:K1 strains, but not O1:K1, were able to cause bacteremia at high frequency. Further experiments suggested that the virulent O7:K1 and O18:K1 strains were able to multiply directly in the bloodstream of the infected animals, whereas almost all O1:K1 bacteria were incapable of such multiplication (24).

The sensitivity of the bacteria to adult rat serum correlated with the lack of virulence (22). In the absence of specific antibodies, virulent O7:K1 and O18:K1 strains were complement resistant, whereas avirulent O1:K1 strains were sensitive. Antibody-independent activation of the classical complement pathway by the O1 antigen seems to be responsible for the complement sensitivity of O1:K1 cells (22, 23). Sialic acid residues seem to impede alternative pathway activation, and strains that possess the K1 (sialic acid) capsule are resistant to killing (22, 23) and opsonization (4, 32, 33) by sera deficient in classical pathway activity.

In human newborns the gastrointestinal tract seems to be the source of *E. coli* which can invade the bloodstream, with the subsequent involvement of the central nervous system (27). Ørskov and Sørensen (20) found that K1 bacteria could be detected in approximately 20% of the fecal samples obtained from healthy babies, and Sarff et al. (27) found *E. coli* K1 in 20 to 40% of the rectal swabs from individuals of all ages. Two-thirds of the neonates born to K1-positive mothers carried K1 strains, and these bacteria tended to be the predominant organism in K1-positive infants (27). Thus, many babies carry K1 bacteria, but only a small percentage of the babies colonized with potentially virulent strains develop bacteremia and meningitis. Because of the low frequency of disease after gastrointestinal colonization and because disease isolates do not seem to differ from fecal isolates of the same serotype (1, 14, 18, 22, 24, 27), it appears that host rather than parasite factors largely determine the outcome. Transplacentally transferred immunoglobulin G (IgG) specific for bacterial antigens may play a crucial role.

Previous protection studies using sera from immunized animals are subject to criticism on the basis that these sera may contain antibodies against more than one type of cell surface antigen. The availability of B-cell hybridomas has brought serology into a new era of precision, and monoclonal technology has already resulted in preparations that have been used in the treatment of patients. It has been shown previously (30) that the injection of monoclonal antibodies to the K1 antigen prevents bacteremia in newborn rats after peroral challenge with O18:K1 bacteria. Antibodies to type 1 pili did not reduce bacteremia significantly (30). Antibodies to *Neisseria meningitidis* B capsular antigen, which is identical to the *E. coli* K1 capsule, protected mice challenged with lethal doses of K1 *E. coli* (5, 17), although very high doses were needed.

In this study we demonstrate that newborn rats can be protected against infection with K1 *E. coli* by antibodies specific for the O-antigen of LPS.

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MATERIALS AND METHODS

Bacterial strains. The *E. coli* strains with sequential numbers 9 (O1:K1), 102 (O18:K1), and 165 (O7:K1) are selected representatives taken from our collection of *E. coli* strains (1) and have been described elsewhere in detail (1, 14, 18, 22, 24).

Test animals. Specific-pathogen-free outbred rats were used in all experiments. Wistar rats were obtained from the Bundesgesundheitsamt, Berlin, Federal Republic of Germany, and Fü albino rats were obtained from the Institut für Medizinische Forschung, Füllinsdorf, Switzerland.

Sera, LPS, media, and buffers. Blood was taken by cardiac puncture, and serum samples were prepared as described previously (22). Samples stored at −70°C were thawed immediately before use. LPS was prepared by the method described by Westphal and Jann (34). The media and buffers used here were as described previously (22).

Bactericidal assay. Bacteria were grown in L broth at 37°C to a density of 10^8 cells per ml, washed with and diluted in phosphate-buffered saline at room temperature, and incubated in 90% serum at 37°C. The viable-cell counts were determined by plating serial dilutions of the reaction mixture on L broth agar plates.

Measurement of hemolytic activity of complement. Hemolytic activity was determined as described previously (15, 22) by hemolysis of sheep erythrocytes sensitized with rabbit anti-sheep erythrocyte serum. Briefly, the serum dilution necessary to yield 50% hemolysis in Veronal buffer (2.5 mM Veronal, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, 75 mM NaCl, 0.1% gelatin, 2.5% glucose; pH 7.5) with 1.25×10^7 erythrocytes in a total volume of 0.25 ml within 1 h of incubation at 37°C was determined. Hemolytic units were defined as the volume of undiluted serum calculated to be sufficient to lyse 0.625×10^7 erythrocytes under these conditions.

Virulence assay. Rat pups were fed 20 μl of bacterial suspensions (2×10^8 cells grown in L broth per ml) with an Eppendorf plastic pipette tip. The rats were returned to the mother rats immediately after feeding. Colonization and bacteremia were scored 3 days after feeding. To detect colonization, the anal area was sampled with sterile wooden toothpicks moistened with phosphate-buffered saline. Blood (100 μl) was taken by cardiac puncture of rats anaesthetized with carbon dioxide vapors. Samples were cultured on minimal agar plates containing meningococcus group B antiserum (1). The purity of bacterial cultures and the identity of K1 bacteria within the samples were established by observing halo formation on these plates.

Hybridoma antibodies. Cells of the *E. coli* O18:K1$^-$ strain A691 (14) were grown in L broth overnight, harvested by centrifugation, washed with and suspended in phosphate-buffered saline, and boiled for 2 h. Outbred rats received five injections (10^9 bacteria in 0.2 ml each) via a tail vein. At 4 days after the last injection, spleen cells were fused with the mouse myeloma line Sp2/0-Ag14 (28) in the presence of polyethylene glycol according to established techniques (7). Supernatants from wells where hybrid cells had grown were screened for anti-O18 antibodies by enzyme-linked immunosorbent assay as described previously (22). Cells from wells yielding positive results were cloned at least twice by limiting dilution or in soft agar. Ten strongly positive clones were selected and further propagated. Enzyme-linked immunosorbent assay and Ouchterlony analysis using class-specific antisera (Sigma Chemical Co., St. Louis, Mo., and Cappel Laboratories, Philadelphia, Pa.) of culture supernatants concentrated by ammonium sulfate precipitation showed that all 10 clones produced IgM(κ) antibodies. Antibodies of the subclone PL2d were used in this study. They were precipitated from culture supernatants with ammonium sulfate and purified by gel filtration (Sephacryl S300; Pharmacia Fine Chemicals, Uppsala, Sweden) followed by ion-exchange chromatography (DEAE-Sephacel; Pharmacia) as described previously (9). The isolated antibodies reacted in agglutination tests with boiled O18:K1 *E. coli* cells at concentrations of 1 μg/ml or higher.

Agglutination assays. Bacteria grown overnight at 37°C in L broth were centrifuged at room temperature, resuspended in the same volume of phosphate-buffered saline, and incubated at 100°C for one h. Gram's safranine solution (E. Merck AG, Darmstadt, Federal Republic of Germany) and formaldehyde (Merck) were added to 3 and 0.5% (vol/vol), respectively. Portions (50 μl) were added to 50-μl samples of serially diluted antisera in U-shaped wells in microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.). The plates were sealed, shaken, and incubated for 16 h at 50°C. Bacterial agglutination was scored as positive when the red bacterial suspension was evenly distributed over the bottom of the wells and negative when a red button was clearly visible.

RESULTS

Passive protection with monoclonal IgM against the O-antigen. Hybridoma cell lines specific for the O18 LPS antigen were isolated and monoclonal rat IgM antibodies were purified to determine whether antibodies to the O-antigen could protect against K1 *E. coli* disease. Protective capacity was tested in the newborn-rat infection model, which shares a number of features with the infection of human newborns including (i) serotype specificity, (ii) the portal of entry and route of dissemination of the bacteria, and (iii) the age dependence of susceptibility of the host (8, 24).

Newborn rats were fed K1 *E. coli* bacteria, and colonization and bacteremia were assayed 3 days later. The effect of anti-O18 IgM was determined by injecting 25, 5, or 0 (i.e., saline) μg intraperitoneally before challenge. The control experiment, in which saline containing no antibody was injected, yielded results similar to those described previously (24), namely that about half of the 5- to 7-day-old rats were bacteremic 3 days after peroral challenge with 4×10^6 O7:K1 or O18-K1 bacteria (Table 1). Only 20% of those rats that had received a 5-μg dose of monoclonal anti-O18 IgM intraperitoneally before bacterial challenge with O18:K1 bacteria developed (low-level) bacteremia ($P < 0.01$). Only 1 of 45 rats that had received a 25-μg dose of IgM yielded a positive blood culture ($P < 0.01$). The protective effect of the anti-O18 monoclonal antibodies was specific since they had no significant protective effect when the animals were challenged with the O7:K1 strain (Table 1).

IgM-mediated bactericidal activity. O18:K1 bacteria were able to multiply for several hours in 90% serum of 7-day-old rats that had received 25 μg of anti-O18 IgM intraperitoneally 2 days before blood was collected by heart puncture (data not shown). It has been shown previously (22, 23) that virulent O18:K1 and O7:K1 cells are resistant to the bactericidal activity of normal adult rat serum and that they are killed (after a delay for unknown reasons) when antibodies directed to the O-antigen are present in the serum. Figure 1 shows the dependence of the bactericidal activity of adult rat serum against O18:K1 cells as a function of anti-O18 IgM concentration. Low concentrations of antibody (<10 μg/ml)

TABLE 1. Protective effect of monoclonal anti-O18 IgM in infant rats challenged orally with O18:K1 or O7:K1 *E. coli*

Challenge strain and injected agent[a]	Bacteremic rats (%)[b]	Significance[c]	Bacteria/0.1 ml of blood[d]
102 (O18:K1)			
Saline	51 (38/74)	$P < 0.01$	20.9
IgM (5 μg)	20 (6/30)	$P < 0.01$	1.7
IgM (25 μg)	2 (1/45)		2
165 (O7:K1)			
Saline	43 (22/51)	$P > 0.2$	14.9
IgM (25 μg)	40 (16/40)		12.4

[a] Injections (100 μl) of saline alone or saline containing rat monoclonal anti-O18 IgM were given intraperitoneally 3 h before the animals were fed the bacterial suspensions.
[b] As determined 3 days after 4 × 10^6 bacteria were fed to 5- to 7-day-old Wistar rats. All animals were colonized.
[c] Calculated by significance of the difference between two proportions.
[d] Geometric mean among samples yielding colonies.

reduced bacterial multiplication, whereas higher concentrations resulted in killing. The observed bactericidal activity reached a plateau at an antibody concentration of about 50 μg/ml. The effect was not caused by agglutination of the bacteria because the addition of antibodies had no effect when heated serum which lacked complement activity was substituted for normal rat serum. Killing of the bacteria starts after a delay of about 1 h (22, 23) and proceeds further between 3 and 4 h (Fig. 1). The addition of anti-O18 IgM (50 μg/ml) to 1-week-old-rat serum had minimal effects on O18:K1 cells. The rate of multiplication was slightly less than that in serum to which IgM had not been added (Fig. 2).

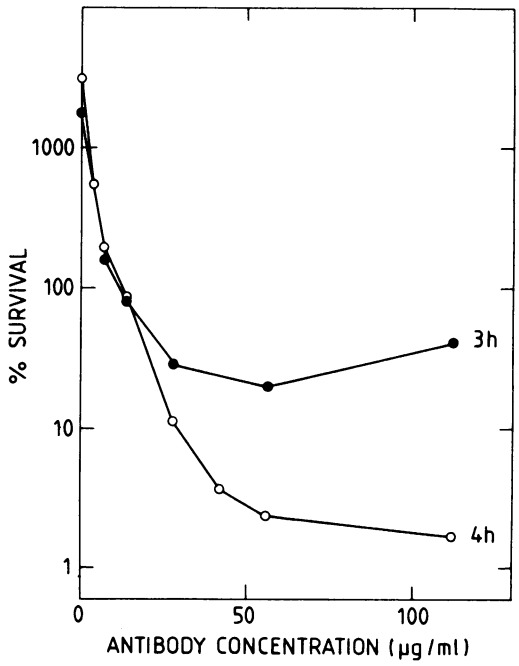

FIG. 1. Effect of monoclonal anti-O18 IgM on the bactericidal activity of adult rat serum. Cells (2 × 10^3) of strain 102 (O18:K1) were incubated in 0.1-ml volumes containing 90% adult Wistar rat serum plus various concentrations of monoclonal IgM. The number of CFU was determined after 3 and 4 h by plating serial dilutions. The percent survival was calculated relative to the initial inoculum.

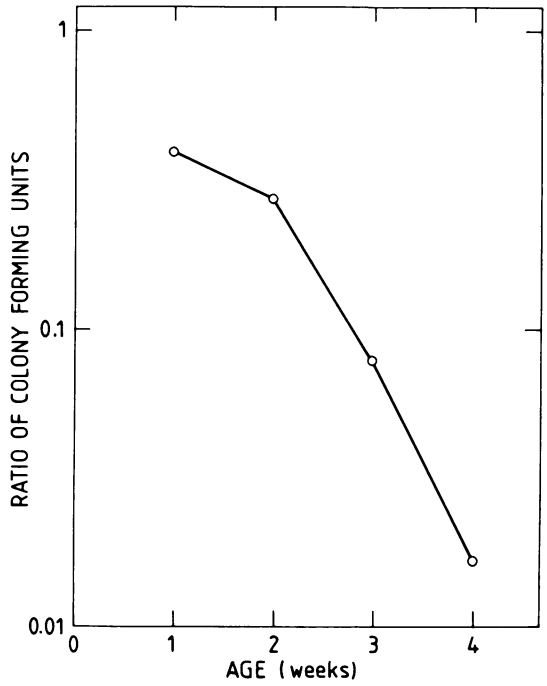

FIG. 2. Age dependence of the anti-O18 IgM-mediated bactericidal activity of rat serum. Cells (2 × 10^3) of strain 102 (O18:K1) were incubated for 3 h in 0.1-ml volumes containing 90% serum and 50 μg of rat anti-O18 IgM per ml (final concentration). Serum pools had been obtained from 1-, 2-, 3-, or 4-week-old Wistar rats (about 30 animals per group). The ratio of CFU found in serum containing monoclonal IgM relative to the CFU in serum with no added IgM is shown.

The effect of antibody addition was more pronounced in the sera of older infant rats, but only in the serum of 4-week-old rats was the number of CFU reduced compared to the initial inoculum. The results indicate that the antibody-mediated direct bactericidal activity of 1-week-old rat serum is not efficient enough to explain the observed protective effect of the IgM injection.

Protection of newborn rats by immunization of the mother rats with LPS. Rats born to mothers that had been immunized twice with purified O18 LPS before term were

TABLE 2. Protection of newborn rats by immunization of mother rats with purified LPS

Challenge strain and injected agent[a]	No. of rats[b]			Rats killed or bacteremic (%)[c]	Significance
	Tested	Killed	Bacteremic		
102 (O18:K1)					
Saline	72	10	23	46	$P < 0.01$
O18-LPS	47	0	0	0	
165 (O7:K1)					
Saline	12	0	8	67	$P > 0.10$
O18-LPS	23	1	16	74	

[a] Fü albino mother rats received two intraperitoneal injections, the first 19 or 20 days before term and the second 7 or 8 days before term. Control animals received 100 μl of saline while the other animals received 100 μl of saline containing 30 μg (first injection) or 100 μg (second injection) of LPS.
[b] As determined 3 days after 4 × 10^6 bacteria were to fed to 4- to 6-day-old Fü albino rats.
[c] Of the surviving animals, all were colonized.

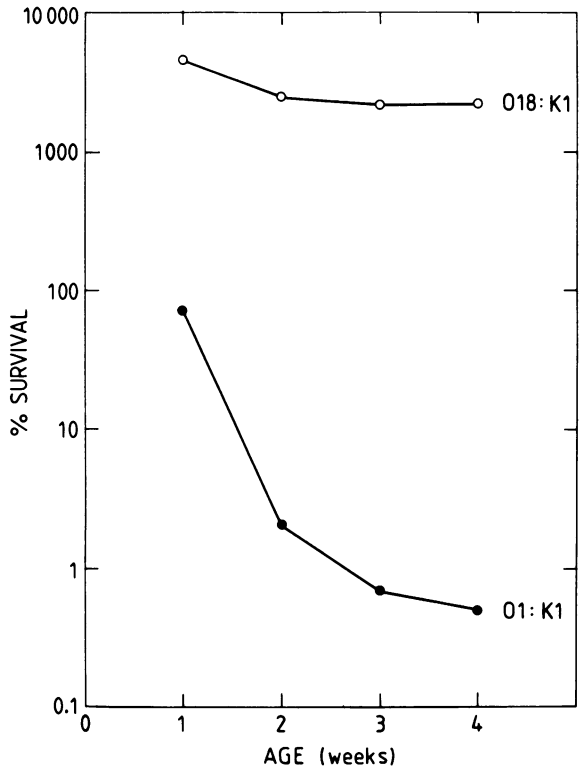

FIG. 3. Age dependence of the antibody-independent bactericidal activity of rat serum. The points indicate the percent survival of O18:K1 (strain 102) and O1:K1 (strain 9) bacteria after 3 h of incubation in 90% Wistar rat serum. The serum pools were the same as those used for Fig. 2.

protected aginst bacteremia and death caused by O18:K1 bacteria (Table 2). Immunization did not interfere with colonization. The protective effect was specific for O18 bacteria, since immunization with O18 LPS did not protect animals that were inoculated with O7:K1 bacteria (Table 2).

Sera from mother rats that had been immunized were strongly bactericidal for O18:K1 cells. Less than 1% of the initial inoculum survived 4 h of incubation in 90% serum. The results obtained with the sera from 7- to 8-day-old animals born to immunized mothers were variable. Although bacteria were able to survive or even multiply in some of the sera up to fourfold within 4 h, up to 10-fold reductions in CFU were found in other sera. Increases of more than 200-fold in the cell numbers were found in sera obtained from pups born to nonimmunized mothers.

Development of the antibody-independent classical pathway bactericidal activity. It has been shown that O1:K1 bacteria are killed by normal rat serum via an antibody-independent activation of the classical complement pathway (22, 23). The bactericidal activity of 1-week-old-rat serum against O1:K1 cells was relatively low (Fig. 3). The number of CFU of the initial inoculum was nearly unaltered after 3 h of incubation in 90% serum. In contrast, serum obtained from 2-week-old or older rats killed these cells efficiently. In contrast to O1:K1 cells, virulent O18:K1 bacteria do not activate complement antibody independently (22, 23) and were able to multiply extensively in nonimmune serum from rats of all ages (Fig. 3).

Age dependence of the classical complement pathway hemolytic activity of rat serum. Studies both in human newborns and laboratory animals indicate that the concentration of a number of complement components is markedly reduced in the sera of newborns (2, 3, 21). Figure 4 shows that the hemolytic complement activity of the serum of 1-week-old rats is about one-third of the adult level. The activity increases rapidly with age in the first 2 postnatal weeks and reaches adult levels at an age of more than 4 weeks. The low bactericidal activity of 1-week-old-rat serum thus correlates with the low hemolytic activity of the serum against sensitized sheep erythrocytes.

DISCUSSION

E. coli is a common cause of meningitis and sepsis in newborns. One capsular and a few O-antigen types predominate among the isolates from cases of meningitis (24, 25). The chemical compositions of both the capsule and the O-antigen seem to determine the degree of direct complement activation and virulence (22–24). In the absence of specific antibodies, the virulent bacteria cause less direct activation of complement than closely related avirulent strains (22, 23, 32, 33).

The ratio of neonates colonized with virulent K1 bacteria to those who actually become infected is high, which may indicate that most newborns are protected by the transplacental transfer of protective antibodies. Despite antimicrobial treatments, the mortality from K1 *E. coli* meningitis remains high (16, 29). Therefore, immunotherapeutic treatments may become important in the management of this disease (5). However, which antigens on the bacterial cell surface are relevant to protective activity? Studies with monoclonal antibodies indicate that at least some of the bacterial outer membrane proteins are not accessible to antibodies on the cell surface of smooth encapsulated *E. coli* bacteria (B. Lugtenberg, personal communication). Furthermore, the presence of antiprotein antibodies does not seem to increase the serum bactericidal activity against K1 *E. coli* (22; P. H. Mäkelä, personal communication).

The K1 capsule, which is structurally identical to the capsules of group B meningococci, is a poor immunogen, and efforts toward the development of a capsular-polysaccharide vaccine have not been very rewarding (5, 26). A high percentage of adults have serum antibodies to the K1 cap-

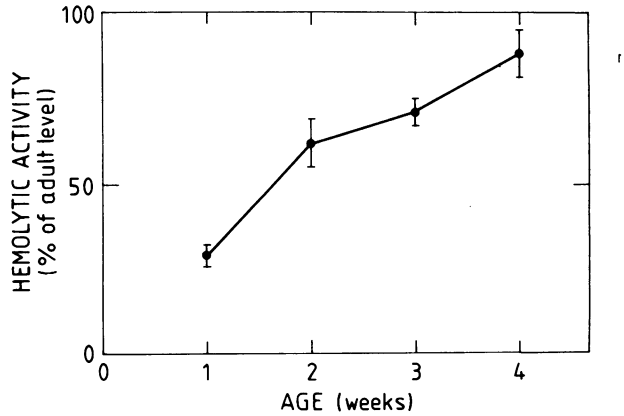

FIG. 4. Age dependence of the 50% hemolytic complement activity of Wistar rat serum. The values are average values derived from three independent experiments and are expressed as a percentage of the values for adult rat serum (average activity, 1,390 U/ml). The vertical lines represent one standard deviation.

sule, nearly all of which are of the IgM class and have relatively low avidity and poor bactericidal activity (5). Monoclonal antibodies to the K1 capsule prevent bacteremia in newborn rats challenged with K1 bacteria (30), and the treatment of neonatal *E. coli* K1 meningitis by the administration of specific anticapsular antibodies has been suggested (5). Glycoproteins of human (and rat) brain do, however, contain oligosaccharide side chains that cross-react immunologically with the K1 capsule (6). Therefore, adverse effects might be caused by a breakdown of immunological tolerance caused by a vaccine or by the transfusion of antibodies.

In this study we demonstrate that the injection of monoclonal antibodies specific for the O-antigen of LPS can protect newborn rats from bacteremia and death caused by K1 bacteria. Specific protection of the pups could also be achieved by immunization of the pregnant rats with isolated LPS. Antibodies against O-antigens of *E. coli* are commonly found in the sera from healthy adults (19; unpublished data) and from newborns (31) but not in the serum of the specific-pathogen-free rats that we used (22). Such antibodies are presumably acquired through natural exposure to *E. coli* bacteria or other organisms which carry cross-reactive antigens. If the results with newborn rats born to immunized mothers can be extrapolated to the situation in humans, one has to assume that most human newborns that are colonized by potentially invasive K1 *E. coli* within the first days of life are protected against sepsis and meningitis by the transplacental transfer of maternal anti-LPS IgG antibodies. Treatment of *E. coli* meningitis with antibodies to the most common O-antigens of virulent K1 *E. coli* might be considered as a future therapeutic option.

Binding of antibody to the bacterial cell surface may initiate complement killing of the bacterium by increasing complement activation or by increasing the bactericidal efficiency of the membrane attack complexes via improved access to suitable membrane sites (10–12). Protection may also be brought about by triggering the binding and ingestion of the bacteria by phagocytes via receptors for the third component of complement or the Fc segment of IgG, or both. The concentration of a number of complement components is markedly reduced in the sera of newborns (2, 3, 21). We have found that the hemolytic complement activity of the serum of 1-week-old rats is about one-third of the adult level. The bactericidal activity of this serum against O1:K1 cells (which activate the classical complement pathway independent of antibody [22, 23]) was relatively low. Whereas adult rat serum killed O1:K1 cells efficiently, the numbers of CFU were almost unaltered after 3 h of incubation in serum from 1-week-old rats. Because the ability to multiply directly in the bloodstream of the infected animals seems to be a crucial property of strains that cause meningitis (24), the observation that O1:K1 cells did not multiply in serum from 1-week-old rats might be a sufficient explanation for the relative avirulence of these bacteria. Alternatively, the complement activation might be protective primarily through opsonization. Comparable results were obtained upon incubation of O18:K1 bacteria in a number of sera that had been collected from 7- to 9-day-old rats whose mothers had been immunized with LPS. The bactericidal activity was low compared to immune adult rat serum, but the bacteria were unable to multiply extensively in these sera, in contrast to the situation with nonimmune serum. In rodents, maternal IgG is actively transmitted to the developing young both before birth via the yolk sac and after birth by receptor-mediated endocytosis across epithelial cells in the small intestine of the young rat (13). The presence of polyclonal anti-LPS antibodies thus seems to limit bacterial growth strongly. In contrast, monoclonal anti-LPS IgM, although protective, only slightly reduced the extensive multiplication of O18:K1 cells in 7-day-old-rat serum. Thus, in this case, complement-mediated opsonization seems more likely than direct killing by the membrane attack complex to account for the strong protective capacity of these antibodies. These results show that the lack of in vitro serum bactericidal activity does not prove the absence of protective antibodies.

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