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Bactericidal Activity of Normal Bovine Serum (NBS) Directed against Some *Enterobacteriaceae* with Sialic Acid-containing Lipopolysaccharides (LPS) as a Component of Cell Wall

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A b s t r a c t

The sensitivity of bacteria to the bactericidal activity of serum depends on the structure and organization of the bacterial outer membrane. Sialic acid has been found in the O-specific region of bacterial lipopolysaccharide (LPS) and it plays an essential role in protecting Gram-negative bacteria against the bactericidal activity of human and animal serum. The susceptibility of Gram-negative bacilli with sialic acid-containing LPS to the bactericidal action of normal bovine serum (NBS) was determined. The examined strains (*Escherichia coli* O104 (PCM 270)*, E. coli* O24 (PCM 195), *E. coli* O56 (PCM 2372), *Citrobacter braakii* O37 (PCM 2346) and *Salmonella enterica* ssp*. enterica* serovar Toucra O48 (PCM 2359) showed variable sensitivity to the bactericidal effect of the serum. The role of the mechanisms of complement activation in the killing process was also determined.

K e y w o r d s: Gram-negative bacteria, lipopolysaccharide, normal bovine serum (NBS), sialic acid

Introduction

The complement system plays an important role in protection higher organisms against bacterial infection. The complement system can be activated in three ways, known as: the classical, lectin, and alternative pathways. The classical pathway plays the most important role in the bactericidal action of serum. The alternative and the lectin pathways are considered to be less important. The latter two pathways can be activated with or without the participation of antibodies. Activation of the classical complement pathway is dependent on the recognition of antigen by IgG or IgM antibodies, whereas activation of the alternative and lectin pathways is not necessarily dependent on the presence of antibody and is therefore a first line of defense for the host organism (Mokracka-Latajka *et al*., 1996; Matsushita *et al.*, 1998; Rautemaa and Meri, 1999).

The sensitivity of Gram-negative roods to the bactericidal action of serum depends on their cell wall structure. The phenomenon of serum resistance of bacteria has a multifactorial basis, and the structure of the outer membrane has an essential role in protecting Gram-negative bacteria against the action of serum. The O-specific side chains of lipopolysaccharides (LPS), capsules and outer membrane proteins (OMP) play a decisive role in this phenomenon (Lachowicz *et al*., 1999; Mielnik *et al.*, 2001; Cisowska and Jankowski, 2004; Bugla *et al*., 2004).

Sialic acids (*N*-acetylneuraminic acid) are important constitutes of glycoconjugates in animal tissues. In eukaryotic cells, sialic acids stabilize glycoconjugates, mediates cell-cell regulation, and regulates transmembrane receptor function (Vimir *et al*., 2004). Sialic acid is not a common component within the bacterial cell, but may occur as a component of the capsule, *e.g.* K1 and K92 antigens in *E. coli,* type III capsular polysaccharides of streptococci of group B, capsules of meningococci of serogroup B, (Egan *et al*., 1977; Marques *et al.*, 1992; Ram *et al*., 1999) or as a component of LPS. The sialic acid has been found in LPS of *E. coli* (serotypes O24; O56; O104) (Jann and Jann, 1977; Orskov *et al*., 1977; Gamian *et al*., 1992;

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Gamian *et al.*, 1994), *Salmonella* Toucra O48 (Kędzierska, 1978; Gamian *et al.*, 2000), *Hafnia alvei* (Romanowska *et al*., 1988; Gamian *et al*., 1991) and *Citrobacter braakii* O37 (Gamian *et al*., 1992a). Serotype O48 *Salmonella* belongs to clinically important bacteria causing intestinal disfunctions and diarrhoea (Nastasi *et al*., 1988; Gamian *et al.*, 2000). The presence of sialic acid in the O-chain of LPS was finally proven in 1991 (Gamian *et al*., 1991). The occurrence of sialic acid in the lipopolysaccharides of the Gram-negative *Enterobacteriaceae* is presented in Table I.

Table I
The occurrence of sialic acid in the lipopolysaccharides in some representatives of *Enterobacteriaceae*

Strain	Structure	References
Escherichia coli O104	→3)βDGal*p*(1→3)βDGal*p*NAc(1→4)αDGal*p*(1→4)αDNeu*p*5,7,9(Ac)$_3$(2→	(Gamian *et al.*, 1992)
Escherichia coli O24	αDGlc*p* ↓ 1, 2 →3)βDGlc*p*(1→3)βDGal*p*NAc(1→7)αDNeu*p*5Ac(2→	(Gamian *et al.*, 1994)
Escherichia coli O56	αDGal*p* ↓ 1, 2 →3)βDGlc*p*(1→3)βDGlc*p*NAc(1→7)αDNeu*p*5Ac(2→	(Gamian *et al.*, 1994)
Salmonella Toucra O48	→3)αLFuc*p*NAc(1→3) βDGlc*p*NAc(1→4)αDNeu*p*5Ac(2→	(Gamian *et al.*, 2000)
Citrobacter braakii O37	→3)LFuc*p*NA*c*(1→3)Glc*p*NAc(1→7)αNeu*p*5Ac(2→	(Gamian *et al.*, 1992a)

In the pathogenesis of Gram-negative bacilli sialic acid plays an essential role in protecting them against the bactericidal activity of serum by inhibition of the alternative pathway of complement activation. The presence of sialic acid on the bacterial surface and its direct interaction with factor H of complement can provide resistance against complement attack (Rautemaa and Meri, 1999). Sialic acid may also contribute to the pathogenicity of bacteria by taking part in epitopes that resemble host tissue components (molecular mimicry). Interesting phenomenon of the molecular mimicry was observed in the case of the LPS of *C. braakii* O37 which shared epitopes with equine and human erythrocytes. This serological mimicry may contribute to the pathogenicity of these bacteria (Gamian, 1996). Gamian *et al*. (1992b) reported that the other erythrocytes tested namely sheep, goat, pig, cat and bovine were not agglutinated by anti-O37 *Citrobacter* serum.

In our laboratory we analyzed the mechanisms of the activation of complement in NBS by *Escherichia*, *Citrobacter* and *Salmonella* strains containing LPS with sialic acid. Our previous results (Mielnik *et al.*, 2001) indicated that the strains of Gram-negative roods possessing sialic acid in the O-specific antigen demonstrate different degrees of sensitivity to the bactericidal activity of normal cord serum which indicates that the presence of *N*-acetylneuraminic acid in LPS does not play a decisive role in determining of bacterial resistance to the bactericidal complement activity of normal cord serum.

The scope of the present investigation was to determine the effect of the presence of *N*-acetylneuraminic acid in the LPS structure on the sensitivity of Gram-negative bacilli to the bactericidal activity of NBS.

Experimental

Materials and Methods

Bacterial strains. The study was carried out on Gram-negative strains which contain sialic acid in the O-specific side chain of LPS. The tested strains were: *Escherichia coli* O104 (PCM 270), *E. coli* O24 (PCM 195), *E. coli* O56 (PCM 2372), *Citrobacter braakii* O37 (PCM 2346) and *Salmonella enterica* ssp. *enterica* serovar Toucra O48 (PCM 2359). The strains were kindly provided by Polish Collection of Microorganisms (PCM) in Wrocław.

Serum. Normal bovine serum (NBS) was obtained from five healthy animals not treated with any antimicrobial drug. The serum samples were collected, pooled and kept frozen (–70°C) for a period no longer than three months. The suitable volume of serum was thawed immediately before use. Each portion was used only once.

Medium. YP broth (bactopeptone, yeast extract and NaCl, pH 7.0) was used as the liquid medium.

Bactericidal activity of NBS. The bactericidal activity of NBS was determined as described previously (Doroszkiewicz, 1997). Briefly, the strains were grown overnight, and then bacterial cells in an early exponential growth phase were transferred to fresh YP and incubated at 37°C for 1 hour. After incubation, the bacterial cells were centrifuged (4000 rpm for 20 min) and suspended in saline. Then the bacteria were mixed with 12.5, 25, 50 or 75% NBS (the serum was diluted with 0.1 M NaCl). Bacteria with serum were incubated in a water bath at 37°C. After 0, 60 and 180 min, samples were collected, diluted, and cultured on nutrient agar plates for 18 h at 37°C. The number of colony forming units (CFU) at time 0 was taken as 100%. Strains, which had a survival above 100% after 180 min of incubation in NBS, were regarded as resistant.

Thermal inactivation of serum. The control was NBS decomplemented by heating the sample at 56°C for 30 min (NBS 56°C).

Treatment of sera. The alternative pathway of complement activation was blocked by incubation of NBS for 20 min at 50°C (NBS 50°C) (Edinger *et al*., 1977). The classical and lectin pathways of complement activation were inhibited using ethylene glycol-bis (β-aminoethyl ether) *N,N,N'N'*-tetraacetic acid (EGTA) (NBS MgEGTA). This effect of preparation was obtained by removing of cations Ca^{2+} from serum by EGTA supplemented with $MgCl_2$. The final concentration of EGTA and $MgCl_2$ in the serum was 10 mmol/l. The EGTA solution was prepared according to Fine *et al*. (1972).

Results

Four serum concentrations (12.5%, 25%, 50% and 75%) were used. The tested strains demonstrated varied resistance to the bactericidal activity of NBS. Two strains: *E. coli* O24, and *E. coli* O104, were resistant to the bactericidal effect of complement protein. *E. coli* O56, *C. braakii* O37, and *S.* Toucra O48 demonstrated higher sensitivity to the bactericidal activity of complement than the *E. coli* O24, and *E. coli* O104 strains, and NBS bactericidal activity was more effective against these strains. To determine the effect of incubation time on killing of the bacterial cell, we determined the survival of bacteria after one and three hours. It was shown that for *E. coli* O56, *Citrobacter braakii* O37, and *S.* Toucra O48 strains, one hour of incubation was sufficient to achieve a high bactericidal effect of the serum. The results concerning the sensitivity of the Gram-negative bacilli with sialic acid-containing LPS to NBS are given in Tables II–VI. As shown in these tables the bacteria proliferated very intensively in the inactivated NBS (NBS 56°C).

In next step we determined the role of the particular mechanisms of complement activation in the killing of Gram-negative bacilli with sialic acid-containing LPS. We examined the biological activity of serum in which the alternative pathway of complement activation was thermally inhibited (NBS 50°C) and serum in which

Table II
Bactericidal activity of NBS against *Escherichia coli* O56

Time of incubation (min)	Serum concentration (%)				
	12.5	25	50	75	$75^{c}_{(NBS\ 56°C)}$
	CFU[a]				
0	69×10^5	29×10^5	21×10^5	NT[b]	81×10^5
60	41×10^4	60×10^3	17×10^2	NT	50×10^6
180	56×10^4	36×10^2	11×10^1	NT	22×10^7

[a] CFU, colony forming units (relevant for Tables II–VI)
[b] NT, not tested (relevant for Tables II–VI)
[c] NBS, decomplemented by heating at 56°C for 30 min (relevant for Tables II–VI)

Table III
Bactericidal activity of NBS against *Escherichia coli* O104

Time of incubation (min)	Serum concentration (%)				
	12.5	25	50	75	$75_{(NBS\ 56°C)}$
	CFU				
0	NT	35×10^4	26×10^5	45×10^5	58×10^5
60	NT	11×10^5	68×10^5	39×10^5	31×10^6
180	NT	51×10^5	13×10^7	15×10^6	37×10^7

Table IV
Bactericidal activity of NBS against *Escherichia coli* O24

Time of incubation (min)	Serum concentration (%)				
	12.5	25	50	75	$75_{(NBS\ 56°C)}$
	CFU				
0	59×10^5	43×10^5	21×10^5	22×10^5	70×10^5
60	35×10^6	13×10^6	27×10^5	18×10^5	38×10^6
180	12×10^7	14×10^7	14×10^6	35×10^6	33×10^7

Table V
Bactericidal activity of NBS against *Salmonella* Toucra O48

Time of incubation (min)	Serum concentration (%)				
	12.5	25	50	75	$75_{(NBS\ 56°C)}$
	CFU				
0	52×10^5	14×10^5	16×10^5	20×10^5	20×10^5
60	23×10^5	84×10^3	65×10^3	39×10^3	14×10^6
180	23×10^5	89×10^3	60×10^3	25×10^3	18×10^7

Table VI
Bactericidal activity of NBS against *Citrobacter braakii* O37

Time of incubation (min)	Serum concentration (%)				
	12.5	25	50	75	$75_{(NBS\ 56°C)}$
	CFU				
0	19×10^5	18×10^5	11×10^5	12×10^5	54×10^5
60	90×10^3	73×10^3	67×10^2	30×10^2	21×10^6
180	71×10^3	18×10^3	57×10^2	29×10^2	57×10^6

Table VII
Bactericidal activity of NBS 50°C[d] and NBS MgEGTA[e] against *Escherichia coli* O56 after 0 min and 180 min of incubation

Time of incubation (min)	Serum concentration of NBS 50°C (%)			
	12.5	25	50	75
	CFU			
0	52×10^5	37×10^5	58×10^5	NT
180	16×10^6	67×10^4	45×10^1	NT
Time of incubation (min)	Serum concentration of NBS MgEGTA (%)			
	12.5	25	50	75
	CFU			
0	85×10^5	94×10^5	87×10^5	NT
180	56×10^5	39×10^5	82×10^4	NT

[d] NBS 50°C, serum with inhibited alternative pathway of complement activation (also relevant for tables VII–IX)
[e] NBS MgEGTA, serum with inhibited classical and lectin pathways of complement activation (relevant for Tables VII–IX)

Table VIII
Bactericidal activity of NBS 50°C and NBS MgEGTA against *Salmonella* Toucra O48 after 0 min and 180 min of incubation

Time of incubation (min)	Serum concentration of NBS 50°C (%)			
	12.5	25	50	75
	CFU			
0	31×10^5	23×10^5	40×10^5	17×10^5
180	43×10^3	30×10^3	75×10^2	93×10^2
Time of incubation (min)	Serum concentration of NBS MgEGTA (%)			
	12.5	25	50	75
	CFU			
0	42×10^5	50×10^5	40×10^5	32×10^5
180	37×10^5	22×10^5	12×10^4	42×10^4

Table IX
Bactericidal activity of NBS 50°C and NBS MgEGTA against *Citrobacter braakii* O37 after 0 min and 180 min of incubation

Time of incubation (min)	Serum concentration of NBS 50°C (%)			
	12.5	25	50	75
	CFU			
0	20×10^5	21×10^5	19×10^5	17×10^5
180	43×10^4	40×10^4	51×10^3	30×10^3
Time of incubation (min)	Serum concentration of NBS MgEGTA (%)			
	12.5	25	50	75
	CFU			
0	35×10^5	36×10^5	27×10^5	35×10^5
180	41×10^4	37×10^4	16×10^4	11×10^4

the classical and lectin pathways of complement activation were blocked (NBS MgEGTA). In this way the study of the bactericidal mechanisms of serum involved determination of the survival of bacteria in sera with certain bactericidal factors removed. In these experiments we used only *E. coli* O56, *S.* Toucra O48 and *C. braakii* O37 which were sensitive to the bactericidal action of complete NBS, because the strains resistant to complete NBS would also be resistant to the bactericidal action of modified serum (Tables VII–IX).

The obtained data indicate the same mechanisms of activation of complement. An important role for the classical and lectin pathways' mechanisms of complement activation was not observed. The dominant mechanism of activation of NBS was the independent activation of complement by the classical, lectin, and alternative pathways, in spite of the fact that all the tested strains have sialic acid in the O-specific antigen.

It was shown that the presence of sialic acid in LPS does not play a decisive role in the determination of bacterial resistance to the bactericidal activity of complement and that the presence of sialic acid in LPS is not sufficient to block of the amplification of the alternative pathway.

Discussion

The differences between rough strains (R form) and smooth strains (S form) in their sensitivity to bactericidal action of serum have been demonstrated many times, including genetic confirmation of LPS O-specific chains participation in protection of the bacterial cell from the bactericidal action of complement (Delabac, 1968; Taylor, 1995). It has been shown (Doroszkiewicz *et al*., 1994; Jankowski *et al*., 1996; Mielnik *et al*.,

2001; Bugla *et al*., 2004) that many smooth strains are sensitive to bactericidal action of complement. Our previous results also confirmed that the sensitivity of Gram-negative bacteria to normal cord serum (NCS) is variable (Mielnik *et al*., 2001).

In the present experiments we used normal bovine serum (NBS). NCS and NBS presented different bactericidal activity. The bactericidal activity of NCS against some Gram-negative bacteria is less efficient than that of NBS. This effect is caused by a deficiency of complement components and a physiological deficiency of IgM antibodies in NCS (Jankowski, 1994; Cisowska and Jankowski, 2004).

E. coli O56 has a small number of repeating units in the O-chains of LPS (Gamian *et al*., 1994) therefore these cells may be more sensitive to NBS than the S forms of *E. coli* O24, *E. coli* O104, *S*. Toucra O48 and *C. braakii* O37 (Gamian *et al*., 1992; Gamian *et al*., 1992a; Gamian *et al*., 1994; Gamian *et al*., 2000). *S*. Toucra O48 and *C. braakii* O37 (S form) were sensitive to the bactericidal action of NBS. The LPS of *S.* Toucra O48 and that of *C. braakii* O37 contain terminal non-reducing sialic acid (Neu5Ac), but the internal sialic acid in *S.* Toucra O48 LPS is 4-substituted, whereas that in *C. braakii* O37 is 7-substituted (Gamian and Kenne, 1993). In the tested strains of LPS endotoxins, except those of *E. coli* O104 the biological O-specific units are terminated at non-reducing end by sialic acid. In *E. coli* O104 antigen the biological O-specific unit has a terminal nonreducing galactose residue (Table I). Immunochemical analysis of the outer membrane showed that the differences in LPS structure could play an important role in determining the susceptibility of Gram-negative strains to the action of serum (Doroszkiewicz *et al.*, 1994; Doroszkiewicz, 1997). The phenomenon of serum resistance of bacteria has a multifactorial basis and the mechanism of bactericidal sensitivity and resistance to the bactericidal action of complement is not fully understood. In conclusion, sialic acid seems to be immunodominant in most of the studied antigens, but the presence of this molecule in LPS is not sufficient for bacterial resistance to bactericidal serum activity.

The *Neisseriae* strains can sialylate their lipooligosaccharide (LOS) and mimic sialylated lactoneoglycosphingolipids, which may serve as a camouflage the bacterium to the host (Moran *et al.*, 1996). Serum-resistant strains of gonococci are known to be more sialylated (Rautemaa and Meri, 1999). Sialic acid, as a component of the cell of these bacteria, plays an essential role in protecting Gram-negative bacteria against the bactericidal activity of serum. Its protective effect is in the enhancement of the binding of factor H to the C3b component of the system which blocks the amplification loop, limiting the activation of the alternative pathway of complement activation. Factor H inhibits the alternative pathway's C3-convertase by dissociating Bb from the C3bBb complex and promotes the inactivation of C3b by factor I (Rautemaa and Meri, 1999). However, Neu5Ac, as a component of capsule K1 of *E. coli* does not interact directly with factor H and the mechanisms of alternative pathway blocking are not inhibited (Meri and Pangburn, 1990).

We determined the role of the particular mechanisms of complement activation in the process of killing Gram-negative bacilli with LPS containing sialic acid. The alternative complement pathway killed all the tested strains without the participation of antibodies. The independent activation of the classical, lectin, and alternative pathways in the serum was observed. This suggests that the tested strains do not have the ability to block the alternative pathway. Devine and Roberts (1994) showed that the possibility of blocking the alternative pathway is perhaps related to the amount of sialic acid on the cell surface. The exact role of sialic acid in LPS is not know. Microbial, immunological, structural and immunochemical studies on these LPS are necessary to understand the role of the sialic acid in interactions of prokaryotes cells with the cells of human and animal hosts.

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