L-Ficolin and Capsular Polysaccharide-Specific IgG in Cord Serum Contribute Synergistically to Opsonophagocytic Killing of Serotype III and V Group B Streptococci

Mioko Fujieda,† Youko Aoyagi,‡ Kousaku Matsubara,§ Yasuhiro Takeuchi,‖ Wakae Fujimaki,‖ Misao Matsushita,‡ John F. Bohnsack,¶ and Shinji Takahashi*†

Divisions of Microbiology and Human Medical Science,‡ Joshi-Eiyoh (Kagawa Nutrition) University, Sakado, Japan; Departments of Pediatrics and Obstetrics and Gynecology,§ Nishi-Kobe Medical Center, Kobe, Japan; Department of Applied Biochemistry, Tokai University, Hiratsuka, Japan; and Department of Pediatrics, University of Utah Health Sciences Center, Salt Lake City, Utah, USA

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Group B streptococci (GBS; Streptococcus agalactiae) are the most common cause of neonatal sepsis and meningitis. Serotype-specific IgG antibody is known to protect neonates against GBS infections by promoting opsonophagocytosis. The L-ficolin-mediated lectin pathway of the complement is also a potential mechanism for opsonization of GBS, because L-ficolin activates the complement after binding to serotype Ib, III, V, VI, and VIII GBS. In the present study, we investigated how L-ficolin and serotype-specific IgG in cord sera contribute to opsonophagocytic killing of GBS. Neither L-ficolin nor serotype-specific IgG concentrations correlated with C3b deposition on serotype Ib and VI GBS, suggesting L-ficolin- and serotype-specific IgG-independent mechanisms of complement activation. The percentage of serotype VIII GBS killed was high regardless of the concentration of L-ficolin and IgG. In contrast, L-ficolin and serotype-specific IgG can each initiate C3b deposition on serotype III and V GBS and promote phagocytosis by polymorphonuclear leukocytes, but L-ficolin and serotype-specific IgG together promote opsonophagocytic killing to a greater extent than does either alone in vitro. This synergy was observed when serotype III-specific IgG concentrations were between 1 and 6 μg/ml and when serotype V-specific IgG concentrations were between 2 and 5 μg/ml. Concentrations of serotype III-specific IgG in cord blood above 7 μg/ml are considered protective for neonates colonized with GBS, but most neonates with IgG levels of less than 7 μg/ml do not develop GBS infections. The data presented here suggest that L-ficolin enhances opsonophagocytosis of serotype III and V GBS when serotype-specific IgG alone is suboptimal for protection.

MATERIALS AND METHODS

Blood. Umbilical cord blood samples were collected from the Department of Obstetrics of Nishi-Kobe Medical Center, Kobe, Japan, and from
TABLE 1 Strains used

<table>
<thead>
<tr>
<th>Serotype</th>
<th>RDP typea</th>
<th>Multilocus sequenceb</th>
<th>Strain</th>
<th>NeuNAc concn (nmol/ml of bacterial suspension at an OD600 of 0.6)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ia</td>
<td>Ia-2</td>
<td>23</td>
<td>I45</td>
<td>15.4</td>
</tr>
<tr>
<td>Ib</td>
<td>Ib-1</td>
<td>12</td>
<td>590448</td>
<td>6.7</td>
</tr>
<tr>
<td>II</td>
<td>II-2</td>
<td>22</td>
<td>Washing</td>
<td>8.7</td>
</tr>
<tr>
<td>III</td>
<td>III-3</td>
<td>17</td>
<td>874391</td>
<td>7.0</td>
</tr>
<tr>
<td>V</td>
<td>V-3</td>
<td>1</td>
<td>116</td>
<td>8.9</td>
</tr>
<tr>
<td>VI</td>
<td>VI-1</td>
<td>1</td>
<td>853009</td>
<td>6.4</td>
</tr>
<tr>
<td>VIII</td>
<td>VIII-1</td>
<td>1</td>
<td>872808</td>
<td>4.2</td>
</tr>
</tbody>
</table>

a RDP, restriction digestion pattern. As described in reference 36.
b As described in reference 13.
c As described in reference 1.

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The percentage of L-ficolin binding was calculated as follows: 100% − the percentage of L-ficolin remaining.

**Measurement of C3b binding to opsonized bacteria.** The opsonized bacteria described above were successively washed twice with TBS-0.1% Tween 20 and three times with TBS and resuspended in TBS-0.02% sodium azide to an OD0.5 of 0.02 by measuring optical density. Individual ELISA plate wells were coated overnight at 4°C with opsonized bacterial samples or with one of four concentrations (12.5, 25, 50, and 100%) of the GBS opsonized with adult serum and 1.6 μg/ml serotype-specific IgG as standards. C3b binding to the bacteria was detected using an anti-C3d MAb (clone 053A-1149.3.1.4; MorphoSys, Oxford, United Kingdom) which binds to C3b, iC3b, and C3d fragments, alkaline phosphatase-conjugated goat anti-mouse Ig (Invitrogen), and the colorimetric substrate p-nitrophenyl phosphate.

**Opsonophagocytic killing assay.** Peripheral blood taken from a healthy donor was heparinized, and PMNs were isolated by gradient centrifugation using Polymorphprep (Axis-Shield PoC AS, Oslo, Norway), washed, and resuspended at 4.6 × 10⁶ cells/ml in RPMI medium 1640 (Invitrogen) containing 0.8% HSA and 20 mM HEPES. The PMN suspension (350 μl) was added to 50 μl of the opsonized bacteria described above and incubated at 37°C for 60 min with shaking. Opsonophagocytic killing of the opsonized bacteria was determined with an optical growth curve assay method as previously described (2, 38) except that 24- and 48-well plates were used to incubate the opsonized bacteria with the PMNs and to measure the growth curves of surviving bacteria, respectively. The percent bacterial survival after opsonophagocytic killing was estimated, and results were expressed as bactericidal indexes (percentages of bacteria killed), which were calculated as follows: 100 − percent bacterial survival.

**Statistical analyses.** The CH50s of each gestational age and adult were compared to each other by one-way analysis of variance followed by a Tukey-Kramer posttest analysis. L-ficolin concentrations of each gestational age and adult were compared to each other by a Kruskal-Wallis test followed by a Steel-Dwass test. Correlations between C3b deposition and factors that contribute to complement activation and between bacterial index and opsonins were assessed by multiple linear regression analysis. P values of <0.05 were considered to be significant.

**RESULTS**

**CH50, L-ficolin concentrations, and serotype-specific IgG concentrations in cord serum.** The CH50 was significantly lower in every gestational age than in adults, and the CH50 in preterm neonates (≤36 weeks gestation) was also significantly lower than that in neonates at 39 and/or ≥40 weeks gestation (see Fig. S1 in the supplemental material). The L-ficolin concentration in some groups of preterm neonates was significantly lower than that in some groups of term neonates and that in adults (see Fig. S2 in the supplemental material). The median concentration of L-ficolin rose markedly at 37 weeks gestation, reaching 83% of that of adults in term neonates. The concentrations of all serotype-specific IgGs were less than 8 μg/ml in approximately 90% of cord sera (data not shown).

Sera for use in experiments were first divided into four categories based on L-ficolin concentration relative to the mean concentration of L-ficolin in term neonates (2.57 μg/ml): (i) sera with L-ficolin concentrations of less than 1.34 μg/ml (more than 1 standard deviation [SD] below the mean), a group which included 91% of sera from preterm neonates, (ii) sera with L-ficolin concentrations of 1.34 μg/ml and 2.57 μg/ml, (iii) sera with L-ficolin concentrations between 2.57 and 3.19 μg/ml (up to 0.5 SDs greater than the mean), and (iv) sera with L-ficolin concentrations greater than 3.19 μg/ml (more than 0.5 SDs above the mean). In each of the four categories of L-ficolin concentrations, serotype-specific IgG concentrations were divided into four categories, as the IgG concentrations were distributed almost evenly over the range, excluding sera with IgG levels greater than 8 μg/ml. From each category (four categories for L-ficolin, with each including four categories for serotype-specific IgG), one serum was selected, and therefore, 16 sera were used to examine the contribution of L-ficolin and serotype-specific IgG to the opsonophagocytic killing of each serotype of GBS. When there was no serum with the appropriate serotype-specific IgG in an L-ficolin category, we selected an alternative serum containing a concentration of L-ficolin that approximated the concentration range for the category (see Table S1 in the supplemental material).

**Amount of L-ficolin binding to opsonized bacteria.** The ability of L-ficolin to bind to bacteria was examined by incubating bacteria with the 16 sera selected as described above. L-ficolin bound significantly to all GBS from all serotypes in a concentration-dependent manner (P < 0.001 for serotypes Ib, III, V, VI, and VIII and P < 0.05 for serotypes Ia and II) (see Fig. S3 in the supplemental material), but L-ficolin binding to serotype Ia and II bacteria was less than 35% even at high concentrations of L-ficolin, a finding consistent with previous observations (1). Serotype Ib, III, V, VI, and VIII bacteria were therefore used to examine the contribution of L-ficolin to opsonization.

**Factors that contribute to C3b deposition on bacteria.** Bacteria were incubated with the 16 sera selected as described above in order to determine which factors contribute to opsonization of serotype Ib, III, V, VI, and VIII bacteria with C3b. The amount of C3b deposition on each bacterium was correlated with L-ficolin concentrations, the amount of L-ficolin binding to bacteria, serotype-specific IgG concentrations, and CH50. The L-ficolin concentration correlated significantly with C3b deposition on serotype III (P < 0.01), V (P < 0.05), and VIII (P < 0.05) GBS. A similar correlation was found between C3b deposition and the amount of L-ficolin bound to the bacteria (data not shown), but the serotype-specific IgG concentration only correlated significantly with C3b deposition on serotype III GBS (P < 0.05) (Fig. 1). Neither L-ficolin nor serotype-specific IgG concentrations correlated with C3b deposition on serotype Ib and V bacteria. CH50 was not correlated with C3b deposition on bacteria of any serotypes tested.

Serotype-specific IgG enhances the alternative-pathway activation on GBS, thereby increasing cell surface deposition of C3b in vitro (37). Bacteria were incubated with serum containing various amounts of serotype-specific IgG (3.3 to 8.9 μg/ml) in the presence of Mg²⁺ and EGTA (which chelates Ca²⁺ required for activation of both classical and lectin pathways) in order to confirm whether serotype-specific IgG in cord serum can enhance alternative-pathway activation. For all serotypes, C3b deposition in the presence of only Mg²⁺ was minimal compared to C3b deposition in the presence of both Mg²⁺ and Ca²⁺. These data indicate that in the absence of intact classical and lectin pathways, cord serum mediates little if any deposition of C3b on bacteria by alternative-pathway activation, even in the presence of considerable amounts of serotype-specific IgG (see Fig. S4 in the supplemental material).

**Factors that contribute to opsonophagocytic killing of bacteria by PMNs.** Bacteria were opsonized by preincubating with the 16 sera selected as described above and were then incubated with PMNs isolated from adult peripheral blood. There was a statistically significant correlation between C3b deposition and bactericidal index (percentage of bacteria killed) for all serotypes
tested ($P < 0.01$ for serotypes Ib and VIII and $P < 0.05$ for serotypes III, V, and VI) (Fig. 2). Serotype-specific IgG concentrations were significantly correlated with the bactericidal index in serotype Ib ($P < 0.05$), III ($P < 0.05$), V ($P < 0.01$), and VIII ($P < 0.001$) bacteria. However, the bactericidal index was very high (similar to that seen with adult serum) in serotype VIII bacteria even when C3b deposition was low or at low concentrations of serotype-specific IgG.
Serotype-specific IgG concentrations did not correlate with C3b deposition in serotype V bacteria (Fig. 1) but did correlate with opsonophagocytic killing (Fig. 2), supporting a model in which L-ficolin initiates C3b deposition on the serotype V GBS by activating the lectin pathway and serotype-specific IgG bound to bacteria acts as an opsonin through binding to the PMN Fc receptor. Both L-ficolin and serotype-specific IgG concentrations correlated with C3b deposition on serotype III bacteria, but a comparison of the coefficients of regression (slopes of the multiple linear regression) indicated that L-ficolin ($P = 0.003$) contributed to C3b deposition more than did serotype-specific IgG ($P = 0.022$) (Fig. 1). On the other hand, serotype-specific IgG ($P = 0.012$) and C3b deposition contributed equally to the bactericidal index in serotype III bacteria ($P = 0.011$) (Fig. 2). These observations also support a model in which serotype-specific IgG binding to bacteria acts not only to activate complement but also as an opsonin through an interaction with the Fc receptor.

**Synergy between L-ficolin and serotype-specific IgG in the opsonophagocytic killing of serotype III and V bacteria.** We used multiple linear regression modeling to determine if the combination of L-ficolin and serotype-specific IgG was likely to be additive or synergistic in its effect on opsonophagocytic killing of serotype III and V GBS. Generally, the bactericidal index increased with increasing concentrations of either L-ficolin or serotype-specific IgG (Fig. 3A). Equations demonstrating a statistically significant relationship between the bactericidal index as the dependent variable and the concentrations of L-ficolin and serotype-specific IgG as the independent variables were derived for both serotypes (Fig. 3A). Synergy between L-ficolin and serotype-specific IgG was estimated by calculating a fractional bactericidal concentration (FBC) index for each serum (Fig. 3B). The FBC was calculated by first calculating the theoretical concentrations of L-ficolin ($X$) and serotype-specific IgG ($Y$) alone that would yield the observed bactericidal index for the serum using the multiple linear regression equation derived in Fig. 3A.

The fractions of the bactericidal index that could then be attributed to each of the factors, L-ficolin and serotype-specific IgG, were then calculated by dividing the actual concentrations of L-ficolin ($x$) and IgG ($y$) by $X$ and $Y$, respectively. The FBC index is the sum of the fractions for L-ficolin alone ($x/X$) and for serotype-specific IgG alone ($y/Y$) and would be equal to 1 if the effects of the two factors were merely additive (4). Instead, the FBC indexes for some sera were less than 0.9 when L-ficolin was 1 to 4 µg/ml and serotype-specific IgG was 1 to 6 µg/ml for serotype III and when L-ficolin was 2 to 4 µg/ml and serotype-specific IgG was 2 to 5 µg/ml for serotype V bacteria (Fig. 3B), indicating that the two factors acted synergistically to enhance opsonophagocytic killing. The synergy between L-ficolin and serotype-specific IgG in opsonophagocytic killing of serotype III and V bacteria was confirmed by using L-ficolin- and serotype-specific IgG-depleted serum to which various concentrations of purified L-ficolin-MASP complex and purified serotype-specific IgG had been added (Fig. 4).

**DISCUSSION**

In this study, we investigated the contribution of L-ficolin and serotype-specific IgG in cord sera to the opsonophagocytic killing of strains of various serotypes of GBS. The contributions of these factors varied between serotypes. L-ficolin and serotype-specific IgG appeared to contribute synergistically in cord sera to opsonophagocytic killing of bacteria when the concentration of serotype III-specific IgG was 1 to 6 µg/ml and when the concentration of serotype V-specific IgG was 2 to 5 µg/ml. Serotype III-specific IgG is mostly IgG2 (9), a subclass that is less effective in activating the classical pathway and binding to PMN Fc receptors (12). Using adult serum, Aoyagi et al. (2) showed that an increase in L-ficolin-mediated opsonophagocytic killing of serotype III GBS is observed when bacteria are preincubated with serotype-specific IgG2 before the alternative pathway is activated.

![FIG 2 Correlations between bactericidal index and C3b deposition (A) and between bactericidal index and serotype-specific IgG concentrations (B). The data for the bactericidal indexes are the means of three experiments for serotype III, V, and VIII bacteria and the means of two experiments for serotype Ib and VI bacteria. Determination coefficients ($R^2$) of overall multiple linear regression are indicated on the left side, and regression coefficients (slopes [s]) of independent variables (C3b deposition and serotype-specific IgG concentrations) are indicated on the graphs. Intercepts of regression lines are not shown. $P$ values of $<0.05$ were considered statistically significant. NS, not significant. Bactericidal indexes of bacteria opsonized with adult serum, which was used as a control, are shown in parentheses.](http://iai.asm.org/.../2017/06/images/fig2.jpg)
but that no increase in killing is observed when bacteria are incubated with the IgG2 anti-serotype III antibody after the alternative pathway is activated, indicating that the increase in killing is due not to Fc receptor-mediated binding to PMNs but to IgG2-mediated alternative-pathway activation. By assessing the inhibitory effects of monoclonal antibodies to FcRII and FcRIII, Noya et al. (31) showed that FcRII is the primary PMN receptor in the phagocytosis of serotype III GBS opsonized with antibody alone whereas FcRII is not necessary in the presence of both antibody and complement when adult serum is used as the source of complement. Two experiments, in the present study and in that by Aoyagi et al. (2), show that C3b deposition is markedly lower on GBS incubated in cord sera than on GBS incubated in adult serum. Deposition of C3b on a serotype III GBS strain that had been opsonized with cord sera was less than 60% of that of the same bacteria opsonized with an adult serum, with the exception of a single cord serum (Fig. 1). This low level of C3b deposition in cord serum compared with that in adult serum is probably a consequence of the low complement activity in cord serum compared to that in adult serum, a result which we demonstrated by measurement of CH50. It is possible that bacteria cannot be opsonized with C3b in cord serum to a level at which the interaction of IgG with FcRII is not needed for opsonophagocytosis. For serotype III GBS, and perhaps for serotype V GBS, opsonization in cord blood sera presumably involves L-ficolin and/or serotype-specific IgG initiating complement activation, leading to C3b deposition, and then both C3b and IgG, bound to bacteria, acting as opsonins. The

![Graph A](http://iai.asm.org/)

![Graph B](http://iai.asm.org/)
bactericidal index was high, however, and serum opsonized the bacteria effectively, independent of L-ficolin and serotype-specific IgG concentrations. This observation may help explain the discrepancy between the high rate of maternal colonization and the low incidence of early-onset disease associated with serotype VIII in Japan (10, 16, 23).

The contribution of L-ficolin and serotype-specific IgG to C3b deposition in cord serum was not clear for serotype Ib and VI bacteria, since neither L-ficolin nor IgG concentrations correlated with C3b deposition despite experimental evidence that L-ficolin can bind to these serotypes and initiate complement activation (1). It is possible that there is another molecule that contributes to complement activation and/or opsonization on serotype Ib and VI bacteria. Mannose-binding lectin, which, like L-ficolin, also initiates the lectin pathway activation, cannot bind to GBS (1), but antibodies directed against GBS cell wall components other than serotype-specific IgG, such as the α and β components of the C protein complex (17, 32), the surface immunogenic protein (Sip) (22), and the surface-bound C5a peptidase (7), may potentially contribute to complement activation and/or opsonization in these circumstances.

The present study is the first report to determine the contribution of L-ficolin and serotype-specific IgG in cord sera to the opsonophagocytic killing of various serotypes of GBS. It is particularly noteworthy that L-ficolin and serotype-specific IgG appear to contribute synergistically to the opsonophagocytic killing of serotype III GBS, because this serotype causes a significant percentage of early-onset diseases, most late-onset diseases in neonates, and the majority of neonatal meningitis cases (33). Lin et al. (20) showed that risk for the early-onset disease caused by serotype III GBS is reduced with increasing serotype III-specific IgG concentrations in cord serum, but both neonates with early-onset disease and neonates colonized by the bacteria at birth who did not develop disease had serotype-specific IgG concentrations that were less than 7 μg/ml. This range of serotype-specific IgG concentrations corresponds to that in some cord sera from neonates whose mothers were immunized with a serotype-specific CPS-tetanus toxoid conjugate vaccine (geometric mean concentration is 7.5 μg/ml) (3). Since the range of serotype-specific IgG concentrations in which synergy between L-ficolin and IgG was observed is similar to that in cord sera from infected and uninfected neonates, relative L-ficolin deficiency may be associated with an increased risk of early-onset disease caused by serotype III bacteria. The present set of experiments, however, did not address this question, which may be difficult to study because the incidence of early-onset disease has been markedly reduced following the widespread introduction of intrapartum antibiotic prophylaxis.

Three single nucleotide polymorphisms (SNPs) in the promoter region and two SNPs in exon 8 of a L-ficolin gene (FCN2) that are associated with marked alterations in L-ficolin concentrations and with ligand binding, respectively, have been described (11, 28). Variations in ligand binding due to this polymorphism may explain the observation that L-ficolin concentrations from some sera bound to bacteria are markedly less than what would be expected from the regression line between concentration and amount of binding to bacteria, but we did not perform SNP analysis on the FCN2 genes in the cord blood samples. The variations in L-ficolin levels and ligand binding associated with these SNPs may also play a role in susceptibility to late-onset neonatal disease for which there are no obvious risk factors except for low levels of serotype-specific IgG (33). Another factor to be considered is that

FIG 4 Correlation between bactericidal index, L-ficolin concentrations, and serotype-specific IgG concentrations for serotype III and V bacteria that were opsonized with L-ficolin- and serotype-specific IgG-depleted serum and various concentrations of the purified L-ficolin-MASP complex and serotype-specific IgG. The opsonization mixtures contained 20% of the L-ficolin and serotype-specific IgG concentrations indicated in the graph. The length of each vertical line indicates the bactericidal index. The data for the bactericidal indexes are the means of two experiments using the same PMN preparation in each bacterium. Open circles indicate an opsonization mixture in which a synergistic effect between L-ficolin and serotype-specific IgG was observed (the FBC index is less than 0.9). The FBC index (k) was calculated as \( k = x/X + y/Y \), where x (L-ficolin) and y (serotype-specific IgG) were the concentrations in each opsonization mixture and X (L-ficolin) and Y (serotype-specific IgG) were the theoretical concentrations at which each alone would yield the observed bactericidal index for the opsonization mixture. These values (X and Y) were estimated from the linear regression equation calculated from the data of mixtures containing the depleted serum and various concentrations of L-ficolin alone and the depleted serum and various concentrations of serotype-specific IgG alone.
some clones of GBS may be more highly associated with neonatal infections. In particular, one serotype III clone, previously identified as multilocus enzyme electrophoresis type 1 (ET1) (29) or restriction digestion pattern type III-3 (RDP III-3) (30, 35) and now designated CC17 by multilocus sequence typing (MLST) (13), seems to have a particular propensity to be associated with both early- and late-onset disease in neonates (5, 18, 21). For the other serotypes, there appear to be one or more clones that are associated with disease and a number of rarer clones that colonize mothers and their babies but that are not usually associated with disease (5). Clinical investigations into the role of L-ficolin in the prevention of neonatal GBS infection should include analysis of the SNPs of the FCN2 gene and MLST of the isolate.

ACKNOWLEDGMENTS

This study was supported by the Promotion Corporation for Private Schools of Japan.

We are grateful to R. Yoshida and T. Yoshida of the Yoshida Clinic, Iruma, Japan, for their contribution of the cord serum collection, T. Hamamoto of the Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan, for providing Cohn fraction III, and F. Gondaira of Denka Seiken, Tokyo, Japan, for providing rabbit antiserum against the serotype-specific CPS.

REFERENCES


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