Inadequacy of Colominic Acid as an Absorbent Intended To Facilitate Use of Complement-Preserved Baby Rabbit Serum in the *Neisseria meningitidis* Serogroup B Serum Bactericidal Antibody Assay

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The surrogate of protection against *Neisseria meningitidis* serogroup B (MenB) is the serum bactericidal antibody (SBA) assay, which measures the functional activity of antibody by using an exogenous complement source. Despite baby rabbit complement having been used in meningococcal serogroup A, C, Y, and W135 SBA assays, it is not recommended for use in the MenB SBA assay due to elevated SBA titers caused by low-avidity anti-MenB capsular antibody in test sera. Therefore, the possibility of absorbing anti-MenB capsular antibody from test sera to enable the use of baby rabbit complement in the MenB SBA assay was investigated by comparing the results with those gained using human complement. Colominic acid from *Escherichia coli* K1, which shares the same linkage residue as MenB polysaccharide, was used as an absorbent due to the commercial unavailability of purified MenB polysaccharide. Inclusion of soluble colominic acid as an absorbent with baby rabbit complement resulted in a general reduction in SBA titers compared with those obtained using baby rabbit complement alone. However, these were not comparable to human SBA titers for all samples. Further optimization and investigations demonstrated that for some samples, colominic acid reduced titers to less than those achieved with human complement, and for others, it was not possible to inhibit titers by using colominic acid. The results suggested that the use of colominic acid will not result in the ability to use baby rabbit complement in the MenB SBA assay, thus not alleviating the difficulties in procuring human complement. However, alternative absorbents, such as purified MenB polysaccharide, may warrant further evaluation.

*Neisseria meningitidis* serogroup B (MenB) remains a major international health problem and cause of meningitis and septicemia. Despite the development of effective capsular polysaccharide vaccines against meningococcal serogroups A, C, Y, and W135 (18), the approach for MenB has been hindered by the poor immunogenicity of the MenB polysaccharide (29) and by fears over the possible induction of autoimmune antibodies (7). Therefore, the development of vaccines which confer protection against MenB disease has concentrated on subcapsular antigens, either singularly or as outer membrane vesicles.

A surrogate of protection against MenB is the serum bactericidal antibody (SBA) assay, with SBA titers having been shown to correlate with the efficacies of outer membrane vesicle vaccines in Norwegian and Cuban teenagers (6, 15). The MenB SBA assay was recently standardized between four laboratories (4) and has been recommended as the primary measure for evaluating candidate vaccine responses without the need for efficacy studies (5).

Early studies using the SBA assay by Goldschneider and colleagues (8, 9) used an exogenous human complement source. However, problems of obtaining human complement of sufficient quantity and quality due to the presence of anti-meningococcal or cross-reacting antibody resulted in the use of a commercially available source as an attractive alternative. Complement-preserved baby rabbit serum (subsequently referred to as baby rabbit complement) has since been utilized in the standardized serogroup A and C SBA assays (23), but its use in the MenB SBA assay has been associated with elevated SBA titers in comparison with those obtained using complement-preserved human serum/plasma (subsequently referred to as human complement) (21, 30). This discrepancy has been associated with the presence of a low-avidity anti-MenB capsular antibody in test sera (21, 30), and for this reason, human complement has remained the preferred complement source for the MenB SBA assay.

Due to the difficulties in obtaining suitable human complement, the possibility of absorbing anti-MenB capsular antibody from test sera (14, 30, 31) to enable the use of baby rabbit complement was investigated. This method would dispense with the need for human complement and have significant cost and standardization benefits. Colominic acid, a capsular homopolymer from *Escherichia coli* K1 which shares the same alpha-(2-8)-linked N-acetyl-D-neuraminic acid linkage residue as MenB polysaccharide, was used due to the commercial nonavailability of purified MenB polysaccharide and as a replacement for the whole cells used in earlier studies (21, 30, 31).

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TABLE 1. SBA titers of antcapsular and PorA MAbs with human and baby rabbit complement, with and without colominic acid (400 μg/ml)

<table>
<thead>
<tr>
<th>Target strain</th>
<th>MAb</th>
<th>Titer*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hSBA</td>
<td>hSBA</td>
</tr>
<tr>
<td>44/76-SL</td>
<td>Anti-MenB</td>
<td>16,384</td>
</tr>
<tr>
<td></td>
<td>capsule</td>
<td></td>
</tr>
<tr>
<td>P1.7</td>
<td>16,384</td>
<td>8,192</td>
</tr>
<tr>
<td>P1.16</td>
<td>1,024</td>
<td>1,024</td>
</tr>
<tr>
<td>NZ 98/254</td>
<td>Anti-MenB</td>
<td>8,192</td>
</tr>
<tr>
<td>capsule</td>
<td>32,768</td>
<td>32,768</td>
</tr>
</tbody>
</table>

* hSBA, SBA titer with human complement; hSBA, SBA titer with human complement, with colominic acid (400 μg/ml) as an absorbent; rSBA, SBA titer with baby rabbit complement; rcSBA, SBA titer with baby rabbit complement, with colominic acid (400 μg/ml) as an absorbent.

MATERIALS AND METHODS

Serum samples. Serum samples (n = 294) were obtained from a phase 1/II study of 75 healthy adults aged 18 to 50 years (26). Briefly, subjects received a three-dose schedule (0, 6, and 12 weeks) of either MenBvac or MenB. Blood samples were collected immediately prior to each dose and 6 weeks following the third dose.

SBA assay. The MenB SBA assay was performed as previously described (4), with minor modifications. Briefly, either human complement (plasma) or baby rabbit complement (Pel Freez, AZ) was used at a concentration of 25% as an exogenous source of complement. SBA titers were expressed as the reciprocals of the final dilutions giving ≥50% SBA killing at 60 min compared with the control group (inactive complement/no test sera) against the MenBvac and MenB vaccine strains 44/76-SL (B:15:P1.7,16) and NZ 98/254 (B:4:P1.7-2,4), respectively.

For absorption studies, colominic acid (Sigma-Aldrich, Dorset, United Kingdom) was added to the bacteriocidal buffer (BB) (4) to give final concentrations of between 400 and 2,000 μg/ml. Sera were incubated with BB-colominic acid for 1.5 to 3 h at room temperature prior to being assayed.

Monoclonal antibodies (MAbs) against the MenB capsule (NIBSC code 95/750) and the PorA subtypes P1.4 (NIBSC code 95/700), P1.7 (NIBSC code 01/514), and P1.16 (NIBSC code 01/538) were obtained from the National Institute of Biological Standards and Control (Hertfordshire, United Kingdom) and assayed as unknown samples in the SBA assay.

Determination of anti-colominic acid IgG and IgM concentrations. Using a subset of samples (n = 36), anti-colominic acid immunoglobulin M (IgM) concentrations were determined using a bead-based assay (17). The methodology was adapted to detect anti-colominic acid IgG concentrations by the inclusion of R-phycocerythrin-conjugated goat anti-human IgG Fcy fragment (Jackson ImmunoResearch Laboratories, PA).

Data analysis. In the SBA assay, titers of <2 were assigned a value of 1 for computational purposes. The relationships between the results gained using the different complement sources (all on a log2 scale) were explored by linear regression analysis, and the Pearson correlation coefficient was determined. Additionally, sample/strain-specific differences in titer levels between results for the different complement sources were determined, and the averages for all samples were calculated.

RESULTS

Following initial optimization of the MenB SBA assay with colominic acid, a final concentration of 400 μg/ml was chosen (data not presented).

Comparison of antcapsular and anti-PorA MAb results gained using human complement and baby rabbit complement, with and without colominic acid. Individual SBA titers gained using human complement (hSBA), baby rabbit complement (rSBA), human complement with colominic acid at 400 μg/ml (hcSBA), and baby rabbit complement with colominic acid at 400 μg/ml (rcSBA) are presented in Table 1 for each of the MAbs. Results from a single run are presented, with almost identical results achieved with two repeat experiments (replicate results were within one SBA titer for each analyte) (data not presented). Inclusion of colominic acid reduced the hSBA and rSBA titers of the anti-MenB capsular MAB to a greater extent than that attributable to assay variation. However, no such reduction was demonstrated with the PorA MAB, demonstrating the inhibition of anti-MenB polysaccharide antibodies by colominic acid.

Comparison of human complement, baby rabbit complement, and baby rabbit complement with colominic acid in the analysis of unknown serum samples. All 294 serum samples were assayed with human complement, baby rabbit complement, and baby rabbit complement with colominic acid at 400 μg/ml. Correlation trend line data from linear regression analysis of results are presented in Table 2 and demonstrate poor correlations between hSBA and rSBA titers due to rSBA titers being generally elevated in comparison to hSBA titers (data not presented). Improved correlations were achieved between hSBA and rcSBA titers due to reductions in rcSBA titers compared to rSBA titers, but they were still poor.

From the raw data, two populations of data were apparent, with the first consisting of samples with low rSBA titers (≤512), where rcSBA titers were similar to hSBA titers, and the second consisting of samples with high rSBA titers (>512), where rcSBA titers were reduced but not similar to hSBA titers.

TABLE 2. Correlation trend line data from linear regression of all results gained using human complement, baby rabbit complement, and baby rabbit complement with colominic acid (400 μg/ml)

<table>
<thead>
<tr>
<th>Target strain</th>
<th>Comparison*</th>
<th>Slope</th>
<th>Intercept</th>
<th>R*</th>
</tr>
</thead>
<tbody>
<tr>
<td>44/76-SL</td>
<td>hSBA vs rSBA</td>
<td>0.05</td>
<td>836.77</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>hSBA vs rcSBA</td>
<td>0.26</td>
<td>45.56</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>rSBA vs rcSBA</td>
<td>0.93</td>
<td>0.14</td>
<td>0.72</td>
</tr>
<tr>
<td>NZ 98/254</td>
<td>hSBA vs rSBA</td>
<td>0.36</td>
<td>117.11</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>hSBA vs rcSBA</td>
<td>0.66</td>
<td>4.81</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>rSBA vs rcSBA</td>
<td>0.62</td>
<td>0.67</td>
<td>0.62</td>
</tr>
</tbody>
</table>

* hSBA, SBA titer with human complement; hSBA, SBA titer with baby rabbit complement; rSBA, SBA titer using baby rabbit complement with colominic acid (400 μg/ml) as an absorbent.

Increased concentrations of colominic acid. Increasing concentrations of colominic acid were investigated with samples for which inclusion of colominic acid at 400 μg/ml had not reduced rcSBA titers to levels comparable to hSBA titers. Table 4 presents the SBA titers for 20 samples and demonstrates that for certain samples, increasing concentrations (up to 2,000 μg/ml) reduced rcSBA titers to levels comparable to hSBA titers. For other samples, increasing concentrations of colominic acid reduced titers only slightly in comparison to
TABLE 3. Average differences in SBA titer steps gained using baby rabbit complement, with and without colominic acid (400 μg/ml), and human complement

<table>
<thead>
<tr>
<th>Target strain</th>
<th>Sample group</th>
<th>Avg difference in SBA titer step (compared to human complement)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>rSBA</td>
</tr>
<tr>
<td>44/76-SL</td>
<td>All</td>
<td>6.69</td>
</tr>
<tr>
<td></td>
<td>rSBA titer of &gt;512</td>
<td>4.63</td>
</tr>
<tr>
<td></td>
<td>rSBA titer of ≤512</td>
<td>4.73</td>
</tr>
<tr>
<td>NZ 98/254</td>
<td>All</td>
<td>4.73</td>
</tr>
<tr>
<td></td>
<td>rSBA titer of &gt;512</td>
<td>3.41</td>
</tr>
</tbody>
</table>

* rSBA, SBA titer with baby rabbit complement; rcSBA, SBA titer with baby rabbit complement, with colominic acid (400 μg/ml) as an absorbent.

Comparison of human complement with and without colominic acid in analysis of unknown serum samples. The effect of colominic acid (400 μg/ml) assayed with human complement was investigated with 58 samples with a range of hSBA/rcSBA titers. The correlation R values, slopes, and intercepts between hSBA and hSBA/rcSBA results were 0.96, 0.888, and 1.022 and 0.94, 0.914, and 0.89 for strains NZ 98/254 and 44/76-SL, respectively. Table 5 presents the sample-specific differences in SBA titers and demonstrates a slight average decrease in titer for human complement with colominic acid in comparison to human complement alone.

DISCUSSION

Absorption of anticapsular or cross-reactive antibody to the MenB capsule has been reported previously (10, 14, 16, 30, 31), but to our knowledge, this is the first report of colominic acid being used as an absorbent with baby rabbit complement in the analysis of sera from a clinical meningococcal vaccine trial. In agreement with previous studies, we have demonstrated that baby rabbit complement generally resulted in elevated MenB SBA titers in comparison to those obtained using human complement (14, 30, 31). Differences between titers obtained with human and baby rabbit complement were subject specific, with samples from the same subject throughout the study generally demonstrating similar differences (data not presented). Furthermore, individual subjects had comparable concentrations of anti-colominic acid IgG and IgM within their four study samples, although these had no correlation with SBA levels obtained using any complement source (data not presented).

Inclusion of colominic acid (400 μg/ml) with baby rabbit complement resulted in a general reduction of SBA titers which was previously demonstrated by Hodge et al., who also demonstrated that this was not due to complement exhaustion or epitope masking (14). Although this observation is not completely understood, it is expected that colominic acid competes for anti-MenB capsular antibodies, which have increased bactericidal activity in conjunction with baby rabbit complement. This was corroborated by the demonstration of the ability of colominic acid to reduce the bactericidal activity of an anti-MenB capsular MAb, but not a PorA MAb, with both human and baby rabbit complement.

Inclusion of colominic acid (400 μg/ml) with baby rabbit complement generally reduced SBA titers to similar levels to those gained with human complement for samples with low rSBA titers (≤512). However, for samples with high rSBA titers (>512), colominic acid resulted in a reduction of titers, but not to similar levels to those achieved with human complement. For samples with rSBA titers of >512, increasing concentrations of colominic acid with baby rabbit complement resulted in differing results. Some samples demonstrated decreases in SBA titers with increasing colominic acid concentrations, suggesting that for these samples colominic acid at 400 μg/ml was not sufficient to absorb all anticapsular antibodies. Other samples demonstrated either little or no reduction in SBA titer with increasing colominic acid concentrations. Previously, Jennings et al. (16) demonstrated the presence of two populations of antibodies in equine antiserum, namely, those

![FIG. 1. Differences in SBA titer steps between results gained using baby rabbit complement, with and without colominic acid (400 μg/ml), and results gained using human complement. (a) Samples with rSBA titers of >512 for NZ 98/254. (b) Samples with rSBA titers of ≤512 for NZ 98/254. (c) Samples with rSBA titers of >512 for 44/76-SL. (d) Samples with rSBA titers of ≤512 for 44/76-SL.](http://cvl.asm.org/Downloaded from November 6, 2017 by guest)
which could be absorbed by MenB polysaccharide and colo- 
minic acid and those which could be absorbed only by MenB 
polysaccharide. It is possible that similar populations were 
present in the human study subjects, with samples where in-
creased concentrations of colominic acid did not increase ab-
sorption being due to an antibody population which may only 
be removed by MenB capsular polysaccharide. This may be 
a result of colominic acid not being a complete antigen (16) 
and therefore not absorbing all anti-MenB polysaccharide 
antibodies. Confirmation that purified MenB polysaccharide 
would absorb greater concentrations of antibody for these 
subjects was not possible due to its commercial unavailability.

Interestingly, for some samples, colominic acid with baby 
rabbit complement resulted in lower SBA titers than those 
achieved with human complement. This was demonstrated 
with colominic acid at 400 µg/ml but was accentuated with 
increasing colominic acid concentrations. Assays of samples 
with colominic acid and human complement demonstrated 
slight reductions in SBA titer for some samples, leading to 
the suggestion of the presence of an anti-MenB capsular antibody 
which is bactericidal with human complement, in agreement 
with the findings of Mandrell et al. (21). However, these minor 
reductions may contribute to but probably do not wholly ac-
count for the large reductions in the presence of colominic acid 
and baby rabbit complement which were demonstrated for 
some samples. It is plausible that for certain samples, colo-
minic acid with baby rabbit complement causes an inhibition 
greater than that attributable to absorption of anticapsular 
antibody. This was particularly demonstrated with increasing 
concentrations of colominic acid, with the mechanism of this 
effect remaining to be established.

The incubation conditions for colominic acid with test sera 
were investigated both during initial optimization and with 
increased concentrations of colominic acid. Incubation at 4°C 
was previously demonstrated to increase binding of anticapsu-
lar antibody to MenB polysaccharide (1, 30). However, we 
demonstrated that temperature did not affect inhibition with 
colominic acid (data not presented), in agreement with previ-
ous reports of Hayrinen and coworkers (11, 12, 13). Increased 
incubation periods and agitation conditions were also investi-
gated but, again, showed no effect on inhibition or SBA titers 
(data not presented).

In this study, we used colominic acid due to its commercial 
availability and promising data from early absorption studies 
(14). Absorption of anti-MenB capsular antibody has also been 
achieved using purified MenB capsular polysaccharide, puri-
ified E. coli K1 capsular polysaccharide, and whole cells from 
both MenB strains and E. coli K1 cells (10, 14, 16, 30, 31). The 
use of whole MenB cells would result in the absorption of 
antibodies against other meningococcal antigens and is there-
fore not suitable for this purpose. Purified MenB capsular 
polysaccharide has been demonstrated to be a more effective 
inhibitor than colominic acid (16, 19) and may be more appro-
priate for future absorption studies. Similarly, purified E. coli 
K1 capsular polysaccharide may also warrant further evalua-
tion. Anticapsular antibody has also been removed from sera 
by attaching the absorbent to microtiter plates (31), resulting 
in the removal of absorbent/antibody from sera prior to assay.

Incorporating soluble colominic acid may have affected our 
results and may explain some of the observed rcSBA titers 
which were lower than hSBA titers. Absorption which results 
in the removal of both the absorbent and the antibody from the 
test sample may be preferential over the soluble methodology 
but may increase the complexity of the SBA assay by adding 
extra steps and costs. However, it should be noted that for the 
pneumococcal serotype-specific IgG enzyme-linked immu-
osorbsent assay, two absorption steps are recommended to 
increase the specificity of the assay (28).

Additional factors other than anticapsular antibody may ac-
count for the differences between results given by the investi-
gated complement sources. These may include certain comple-
ment down-regulatory proteins which may enhance serum 
resistance due to species specificity in binding to meningococci.

Human C4b-binding protein, but not that of baby rabbits or 
rodents, binds to gonococci, resulting in resistance to comple-
ment-mediated lysis by the classical pathway (25). Similarly, 
human factor H, but not those of other species, binds to factor 
H binding protein (previously called genome-derived neisserial 
antigen 1870) (24), enhancing resistance to complement-me-
diated lysis by the alternative pathway (20, 27). Such differ-
ces may be partially responsible for the higher titers 
achieved with baby rabbit complement than with human com-
plement. However, for some samples, results obtained with 
human complement were either identical to, similar to (within 
assay variation), or even greater than those given with baby 
rabbit complement (data not presented). Furthermore, anti-
capsular and PorA MAbs gave almost identical titers with 
human and baby rabbit complement. This may suggest a sam-
ple-specific effect or may be due to the inclusion of heparin in 
the BB (due to the use of plasma as a human complement 
source), resulting in inhibition factor H (2, 3). Nonetheless, it 
has been demonstrated that heparin at the concentration used 
does not affect titers in the MenB SBA assay (4, 22), suggesting 
that binding of factor H was not sufficient to affect the outcome 
of the MenB SBA assay.

Most current candidate vaccines designed to confer protec-
tion against MenB disease are based upon target antigens 
other than the MenB capsule, and therefore absorption of 
anticapsular antibody should not interfere with the evaluation 
of SBA responses to these vaccines. Conversely, for certain 
vaccines, the response against the capsule may be important, 
and therefore the absorption of anticapsular antibody is not 
recommended in their evaluation.

In summary, colominic acid reduced rSBA titers, but

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**TABLE 5. Differences in SBA titers gained using human complement with and without colominic acid (400 µg/ml)**

<table>
<thead>
<tr>
<th>Titer step difference (compared to hSBA titer)*</th>
<th>% of samples with titer step difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NZ 98/254</td>
<td>44/76- SL</td>
</tr>
<tr>
<td>-3</td>
<td>0.1</td>
</tr>
<tr>
<td>-2</td>
<td>0.2</td>
</tr>
<tr>
<td>-1</td>
<td>0.3</td>
</tr>
<tr>
<td>Equal</td>
<td>0.4</td>
</tr>
<tr>
<td>+1</td>
<td>0.5</td>
</tr>
<tr>
<td>Avg difference in SBA titers</td>
<td>0.6</td>
</tr>
</tbody>
</table>

|                  | 0.7                                  |
|                  | 0.8                                  |

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* hSBA, SBA titer with human complement.
these were not comparable to those gained using human complement for all samples. These results suggest that colo-
monic acid will not enable the use of baby rabbit comple-
ment in the MenB SBA assay, thus not alleviating the issues of acquiring suitable human complement. The use of puri-
fied MenB capsular polysaccharide was not investigated dur-
ing this study and may be an alternative which warrants further investigation. Furthermore, alternative complement sources, such as bovine sources (5), may be more appropri-
ate, and further evaluation is awaited. However, successful
large-scale comparisons between complement sources will be required prior to the use of nonhuman complement and any absorbent in any future vaccine trials.

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ingococcal B vaccines in adolescents]). We thank Sarah Frankland from the Vaccine Evaluation Unit for determination anti-colicomic acid IgG and IgM concentrations.

REFERENCES


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