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Comparison among Opsonic Activity, Antimeningococcal Immunoglobulin G Response, and Serum Bactericidal Activity against Meningococci in Sera from Vaccinees after Immunization with a Serogroup B Outer Membrane Vesicle Vaccine

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Opsonic activity in sera from 27 military recruits vaccinated with the Norwegian meningococcal serogroup B outer membrane vesicle vaccine was measured as respiratory burst with polymorphonuclear leukocytes as the effector cells and meningococci of the epidemic strain as the target. The results were compared with antimeningococcal IgG antibodies against an outer membrane vesicle coat in an enzyme-linked immunosorbent assay and with serum bactericidal activity. The vaccinees were immunized twice, with a 6-week interval between the two. The serum samples studied were collected at day zero, after 6 weeks, and after 12 weeks. Both serum bactericidal activity and respiratory burst were measured by adding external serum as the complement source. The results revealed a significant increase in specific IgG response, serum bactericidal activity, and respiratory burst after vaccination. We found a highly significant correlation between the responses in all three assays ($P < 0.0001$). The highest correlation was found between respiratory burst and antimeningococcal IgG response ($r = 0.93$). This result strongly indicates that respiratory burst is mediated almost exclusively by IgG antibodies. The correlation between antimeningococcal IgG response and serum bactericidal activity was slightly lower ($r = 0.83$). The correlation between respiratory burst and serum bactericidal activity was further reduced ($r = 0.78$), and some of the sera revealed a marked preference for only one of the activities. This result means that respiratory burst and serum bactericidal activity in part are induced by different mediators, and to obtain a more complete picture of the potential protective activity, both assays should be applied to survey a vaccine trial.

Specific antibodies and a functional complement system are of crucial importance in the host defense against systemic meningococcal infections. Serum bactericidal activity (SBA) correlates with protection as shown in several clinical studies (13, 32). Consequently, patients with defects in the terminal complement pathway are highly susceptible to meningococcal disease, especially from meningococci of uncommon serogroups (8). However, the presence of serum opsonins to facilitate phagocytic killing is also likely to be of great importance in the in vivo defense against this organism. Ross et al. (31) have shown that serogroup B meningococci are more resistant to bactericidal killing than serogroup A and C meningococci but are highly susceptible to killing by polymorphonuclear leukocytes (PMN) after opsonization. Furthermore, a human monoclonal antibody against meningococcal group B polysaccharide that is highly opsonic but not bactericidal and that confers high protection in animal models has been produced (27).

Phagocytosis and SBA are triggered by very similar mediators: antibodies and complement. Immunoglobulin G (IgG) and IgM antibodies activate complement very efficiently and may lead to direct bacteriolysis, as seen in the bactericidal assay. However, during complement activation, C3 and C4 split products will be deposited on the bacterial surface, and these fragments may be effective opsonins. IgG antibodies bound to bacteria are also excellent opsonins, and a synergistic opsonic effect is achieved when the target is covered with both IgG and complement split products. PMN and macrophages constitutively express Fcγ receptors (FcγRs) (37) and complement receptors (30). An immune response in which the effector function is biased against phagocytosis rather than bacteriolysis may be beneficial to the host, since intracellular destruction will minimize intravascular release of bacterial endotoxin and thus reduce the risk for septic shock (21).

The high incidence of meningococcal disease from serogroup B meningococci in Norway (23) has motivated the development of an outer membrane vesicle (OMV) vaccine based on a representative *Neisseria meningitidis* strain from the epidemic. Several clinical trials have been performed (2, 3). Detection of meningococcus-specific IgG antibodies (and IgA and IgM antibodies) against meningococcal outer membrane protein antigens by an enzyme-linked immunosorbent assay (ELISA) (28, 29) and measurements of SBA (9, 19) have been used to evaluate immune responses in these vaccine trials.

We have previously established a flow cytometric assay to measure respiratory burst (RB) in PMN with meningococci as the target cells (1). RB is a terminal effect of phagocytosis thought to reflect microbial killing. We use serial dilution of complement-inactivated test sera and add a constant amount

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of an external complement source to each dilution. By this means we may easily quantitate the opsonic activity in individual serum samples.

The aim of this study was to use vaccinee sera from one clinical trial (phase II-2) (2) to measure RB by this new method and see if this assay might be used as a test to study the immune response after vaccination. The RB results were compared with the IgG response against meningococci and with the SBA. A brief presentation of the IgG and bactericidal data has been published previously (18, 19, 29).

MATERIALS AND METHODS

The vaccine. An OMV preparation from the *N. meningitidis* 44/76 (B:15:P1.7,16:L3,7,9) adsorbed to aluminum hydroxide at a protein:aluminum hydroxide ratio of 1:67 (wt/wt) was prepared as described previously (11).

Study subjects. Twenty-seven volunteer military recruits aged 19 to 20 years received two vaccine doses, 25 μg of protein each of the OMV vaccine (3). The vaccine was injected as two 0.5-ml doses intramuscularly in the deltoid muscle, with the doses given 6 weeks apart. Sera were collected at different time points, and the results presented in this study are from sera collected just before vaccination, after 6 weeks (time of second dose), and after a total of 12 weeks (6 weeks after the second dose). Tonsillopharyngeal swabs were collected at weeks 0, 6, and 12 and tested for growth of *N. meningitidis* and the serogroup, serotype, and subtype were determined (6, 40).

PMN. Venous blood from healthy adult volunteers, heterozygous for the $Fc\gamma RII^{LRHR}$ allotype (38) (kindly typed by J. G. J. van de Winkel, Utrecht, The Netherlands) was drawn into heparinized vacuum tubes, and the erythrocytes were lysed with a solution containing 8.3 mg of NH_4Cl per ml, 1 mg of $NaHCO_3$ per ml, and 0.08 mg of EDTA (pH 6.8) per ml. The leukocytes were washed twice with Hanks balanced salt solution (HBSS) (Gibco Laboratories) with 0.2% bovine serum albumin (BSA), and the cell concentration was adjusted to 5×10^6/ml. No additional purification of PMN was required, because further analyses were done by flow cytometry that easily discriminates PMN from other cells within the suspension.

Complement source for RB. Normal human serum from one healthy donor with no detectable antibody against *N. meningitidis* 44/76-SL was used as the source of complement for opsonization of meningococci. We found no antibodies against meningococcal antigens measured at serum dilutions starting at 1:20 in an ELISA with whole-cell *N. meningitidis* as the antigen and developed with an alkaline phosphatase-conjugated anti-IgG. The complement source also did not give any measurable RB to the 44/76-SL strain. The serum was aliquoted in small volumes, stored at −85°C, and thawed immediately before use.

Bacteria to be used in RB assay. *N. meningitidis* 44/76-SL (B:15:P1.7,16) was grown as described previously for SBA (19), fixed in 70% ethanol overnight at 20°C, and washed in HBSS. The concentration was adjusted to 10^9 bacteria per ml, aliquoted in 1-ml ampoules, and stored at −85°C.

RB. The RB analysis was performed mainly as described previously (1). All sera to be tested were heated to 56°C for 30 min to inactivate endogenous complement. Fifty microliters of a threefold dilution of sera was mixed with 5 μl of meningococci (10^9 bacteria per ml) in U-bottomed microtiter plates and incubated for 30 min at 37°C with continuous agitation. Then, 5 μl of serum as the complement source was added, and the incubation continued for 8 min at 37°C with agitation. Each dilution was tested in duplicate. As an indicator for RB, we used the nonfluorescent probe dihydrorhodamine 123 (DHR) (Molecular Probes, Eugene, Oreg.) that will be oxidized to fluorescent rhodamine 123 during RB (33). A stock solution of DHR was prepared by dissolving 10 mg in 1 ml of dimethyl sulfoxide. This solution was aliquoted and stored at −85°C until use. DHR was added to the effector cells to give a final concentration of 10 μg/ml just before mixing 50 μl of the effector cells with the opsonized bacteria, and the incubation continued for 8 min at 37°C with agitation. Each dilution was tested in duplicate. The reactions were stopped by placing the microtiter plates in an ice bath until RB was measured by flow cytometry.

Flow cytometry. Flow cytometry was performed as previously described (1). Briefly, the samples were run on an EPICS Profile II flow cytometer (Coulter Electronics, Luton, Bedfordshire, United Kingdom) with an 15 mW argon laser. The excitation wavelength was 488 nm, and standard Coulter filters were used in all measurements. On the scatter histogram, a gate was set on the PMN, and RB was measured as percent positive cells within the gate with three-decade logarithmic amplification on the fluorescence detector. By this method PMN could easily be discriminated from monocytes and lymphocytes (and bacteria) without any further purification of the leukocyte fraction. As a negative control, the test serum was omitted and replaced by HBSS containing BSA. This mixture was used to set the correct region on the fluorescent axis. About 3,000 effector cells were counted in each sample, and all determinations were performed in duplicate. The results are presented by summarizing the percent RB positive PMN at each dilution (starting at 1/6). Activities below 10% at any dilution are defined as zero.

Antimeningococcal OMV ELISA. The ELISA was performed as described previously (17) using OMV coat from strain 44/76 (B:15:P1.7,16). Specific IgG was detected with alkaline phosphatase-conjugated swine anti-human IgG antibodies (Orion Research, Inc.). All ELISA results are scaled relative to an internal standard in arbitrary units (units per milliliter) (29).

SBA. The SBA assay was carried out as described earlier (9, 19) with 30 min of incubation at 37°C. Sera were tested in a twofold dilution series starting at 1:2. A 25% final dilution of a selected human serum served as an external complement source. The complement source had very low activity in OMV ELISA and no detectable bactericidal or immunoblotting activity (39) against the 44/76-SL strain. SBA titers are given as reciprocal $\log_2$ values of the dilution giving at least 50% killing of the inoculum measured as colony-forming units.

Statistical methods. The relationship between the different assays was analyzed by linear regression analysis, and Pearson's linear correlation coefficient was determined. Differences between paired samples were evaluated by the Wilcoxon signed rank test, and P values of <0.05 were considered significant.

RESULTS

Immune response after vaccination. The immune response to strain 44/76-SL after vaccination was measured in three different assays: specific anti-OMV IgG antibodies, SBA, and PMN-mediated RB. Sera taken from three different time points (prevaccination, 6 weeks after the first dose, and 6 weeks after the second dose) were analyzed. The results revealed a large variation in activities between the individual serum samples, both in the prevaccinated and postvaccinated sera. However, a significant increase in serum response against strain 44/76-SL after immunization was revealed in all three assays (Fig. 1): there was a marked increase in anti-OMV IgG response after the first dose ($P < 0.0001$) and also after the second dose ($P < 0.005$) (Fig. 1A). The increase in SBA after vaccination was also more pronounced after the first dose ($P < 0.001$) than after the second ($P < 0.005$) (Fig. 1B), and similar responses were seen for RB ($P < 0.0001$ and $P < 0.005$, respectively) (Fig. 1C). However, one of the vaccinees responded very weakly, although a small response was noted after 12 weeks. Three of the vaccinees did not receive the second dose, and their activities increased but not as much as the activities of vaccinees who received the second dose. Almost half of the recruits were asymptomatic but had *N. meningitidis* in the throat at the time of vaccination, and these meningococcal carriers had higher prevaccination levels of anti-OMV IgG antibodies, SBA, and RB activity than noncarriers. Eight of the carriers were colonized by nongroupable strains, three by group B and one by group W. However, there was no correlation between carrier groups and the immune response against strain 44/76 (group B) that was used as the target strain in all three assays. Some of the prevaccinated carriers were also colonized by new strains during the trial, whereas five vaccinees became carriers after the trial had started.

Correlations between the assays. To test for any correlations between the assays, we did linear regression analysis on the results from two and two assays and calculated the correlation coefficients. By this method, we found a very high correlation between anti-OMV IgG antibodies and RB ($r = 0.93$, $P < 0.0001$) (Fig. 2A), whereas the correlation between IgG and SBA was somewhat lower ($r = 0.83$, $P < 0.0001$) (Fig. 2B). Also we observed a significant correlation ($r = 0.78$, $P < 0.0001$) between SBA and RB (Fig. 2C).

Performance of the RB assay. Each serum sample was diluted threefold, and each dilution was measured for RB activity. RB results were calculated from the area under the histogram bars where each dilution is plotted on the abscissa and the percent fluorescence-positive PMN is plotted on the ordinate (Fig. 3). The percent positive PMN at each dilution is summarized to give ΣRB%. However, the results might also be

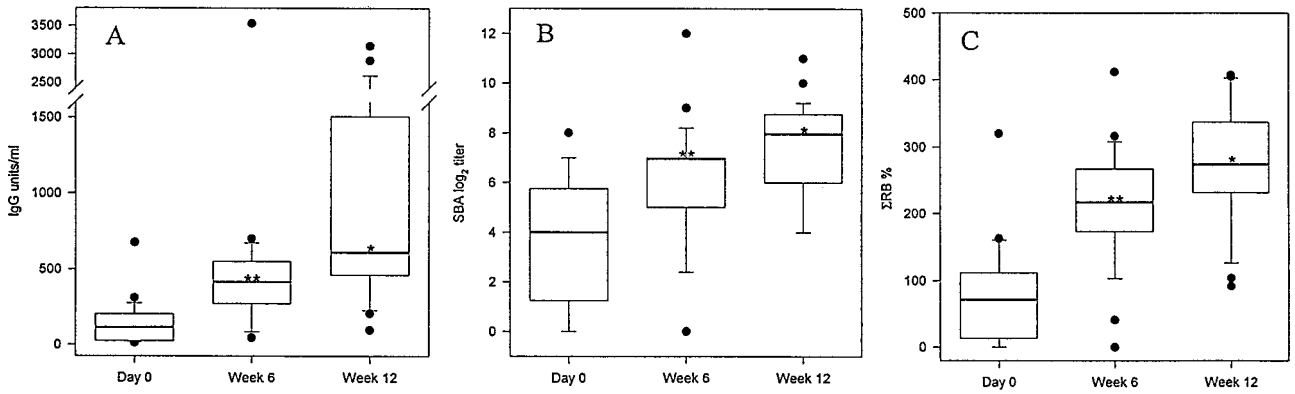


FIG. 1. Box plots showing the immune responses as measured by anti-OMV IgG antibodies (A), SBA (B), and RB (C) against strain 44/76-SL. The boxes indicate the 25th to 75th percentile values, the error bars indicate the 10th to 90th percentile values, the horizontal thick line denotes the median value, and the solid circles denote values outside the 10th to 90th percentiles. Only data from vaccinees for which all three serum samples were available are included in the plot. The IgG values are given as arbitrary units per milliliter, the SBA values are given as reciprocal $\log_2$ of the serum dilution, and the RB values are given as ΣRB%. Values that had increased significantly from the previous sample are indicated ($P < 0.005$ [*] and $P < 0.0001$ [**]).

presented as a titer, given by the reciprocal value of the highest serum dilution giving positive RB. The former presentation is used in this paper because this gives a better discrimination between samples than the corresponding titer (Fig. 3). The correlation to anti-OMV IgG antibodies remains unchanged by either of these two methods, whereas the correlation to SBA decreased from 0.78 to 0.67 when the RB results were given as titers (data not shown).

To test for interassay and day-to-day variations, two control serum samples were included in each assay. The results from these control sera demonstrated high reproducibility: the high-titer serum revealed a mean RB response of 258% (±13.4%) and the low-titer serum revealed a mean RB response of 18% (±8.8%) (standard deviations given in parentheses). Also, when test sera were reanalyzed, they proved to be reproducible within this range. The difference between duplicates was regularly less than 5%.

DISCUSSION

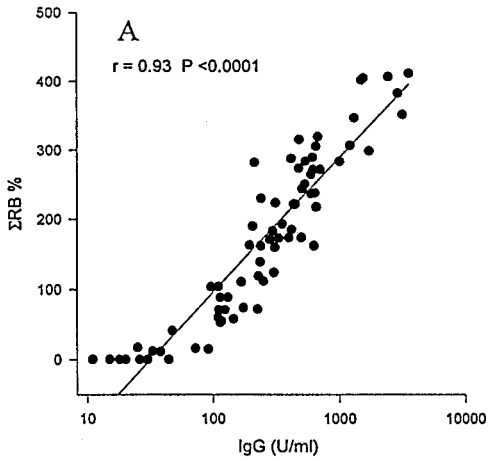
Both SBA and opsonic activity are crucial for the protection against meningococcal disease, although the former appears to be the best documented (13). Test procedures for in vitro SBA are well established in many laboratories. Routine assays to measure phagocytosis are more uncommon, as they may be more cumbersome technically and may be more difficult to reproduce. The method we have developed is easy to perform and proved highly reproducible. In this study we used this RB method on clinical vaccination material and compared the RB results with the specific antimeningococcal OMV IgG antibodies and SBA results.

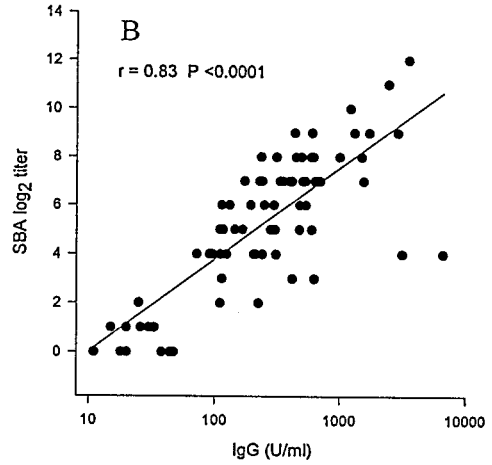
The high prevalence of carriers among the military recruits makes this group difficult to use for evaluation of vaccine effects. The increased immune response against meningococci may, in part, be effected by interpersonal spread of bacteria rather than a response to the vaccine. However, the vaccine effect has been well documented previously (19, 29) and was not a prime aim of this study. From this rather small number of vaccinees, when tested against the 44/76 strain, we could not find any higher reactivity in sera from subjects colonized with group B meningococci than from those colonized with other groups.

We observed a very high correlation between anti-OMV IgG antibodies and RB ($r = 0.93$). This correlation is also in accordance with previous studies (22, 34) and strongly suggests that IgG antibodies are the prime inducers of RB. However, complement is required, because heat inactivation of the complement source reduces activity dramatically (data not shown). The correlation between anti-OMV IgG antibodies and SBA was somewhat lower ($r = 0.83$), and the correlation between SBA and RB, although highly significant, was further reduced ($r = 0.78$), indicating that SBA is differently influenced by serum factors than is RB. This is clearly illustrated in Fig. 3C, as several serum samples reveal high RB and low SBA and vice versa. Similar effects have been observed in several experiments using murine and even human monoclonal antibodies: some monoclonal antibodies may be strong opsonins without giving any bactericidal effects (7, 27; also unpublished observations from our laboratory). Such sera with an almost inverse correlation between RB and SBA may be valuable tools to delineate the different requirements for inducing either of these two responses. Possibly, some of the sporadic incidences of vaccine failure might, in part, be explained by such individual variation in effector functions.

RB and SBA are related effector functions, as both are induced by antibodies and complement. However, SBA depends on activation of the entire complement cascade through C9 for the generation of the membrane attack complex that subsequently causes membrane leakage. Complement-mediated opsonization needs only activation through C3 to cause deposits of C3b, iC3b, and C4b that then ligate to the corresponding receptors on phagocytes. A synergistic opsonic effect is achieved when the phagocytes are triggered through both complement receptors and FcγRs.

The potential for an antibody to bind FcR or activate the complement cascade is strongly dependent of the antibody isotype. IgM antibodies are powerful activators of the complement cascade, but they probably cannot induce RB by FcR binding, as there are few FcμRs on neutrophils. Also, the different IgG subclasses reveal individual patterns for these effector functions. IgG1 and IgG3 both bind to FcγRs and activate complement readily (1, 12, 24, 37), whereas IgG2 reveals a more restricted FcγR binding (5, 26) and only activates complement under certain conditions (1, 24). A detailed study concerning antibody isotype against meningococci in





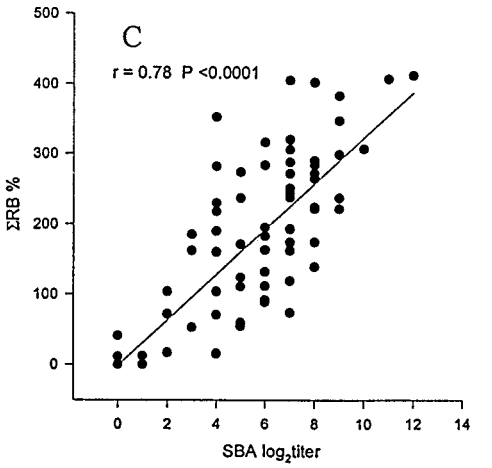


FIG. 2. Linear regression curves showing the correlations between the different assays. The correlation between the anti-OMV IgG response and RB (A), between the anti-OMV IgG response and SBA (B), and between the SBA and RB activity (C) are shown.

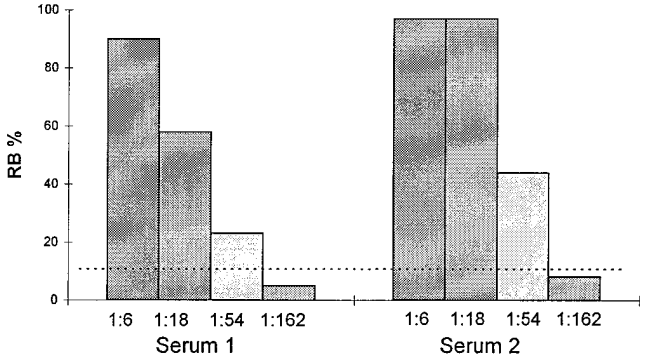


FIG. 3. Bar histogram illustrating the two different modes of presenting the RB data: the histograms shows the RB activity at each dilution tested in serum 1 and serum 2. If 10% is used as the cutoff (dotted line), both samples give identical titers of 54. However, when we summarize the responses at each dilution, the two samples give different results: serum 1 gives a ΣRB% (90% + 58% + 23%) of 171%, and serum 2 gives a ΣRB% (97% + 97% + 44%) of 238%.

these sera is put forward in our laboratory, and these results may help to uncover some of the detailed inducer mechanisms for RB and SBA.

One major difference between the assays for SBA and RB is that we use ethanol-fixed bacteria in the RB assay. This treatment may disrupt certain epitopes and may explain some of the discrepancies between the assays. However, the broad specificity of polyclonal serum antibodies may render single epitopes less important in the overall activity. The use of alcohol-killed bacteria enables us to avoid daily cultivation and the potential spread of hazardous bacteria. By growing a large bulk of bacteria that is fixed and aliquoted, these bacteria can be used through the entire study, thus minimizing any day-to-day variations of the meningococci (36).

In the SBA assay the complement source is used at 25% dilution, whereas in the RB assay we use a 10% dilution. This will favor a contribution from the alternative complement pathway in SBA compared with RB and may explain some of the differences between these assays.

Some reports have described antibodies that act as inhibitors for SBA. Monoclonal IgG antibodies against class 4 outer membrane protein have been reported to block bactericidal killing (25), and IgA antibodies against the polysaccharide capsule may inhibit the IgG-mediated lysis of meningococci (20). There has been some concern about the occurrence of such epitopes in a vaccine formulation, so it would be interesting to study whether analogous effects could be obtained in RB assays.

Several protocols to measure opsonophagocytic activity against meningococci in clinical samples have been described: Sjursen et al. (34, 35) used flow cytometry to measure phagocytosis of fluorescein isothiocyanate (FITC)-labelled meningococci, and several reports (10, 14–16) describe chemiluminescence methods to measure the production of reactive oxygen intermediates generated during phagocytosis. All these reports use the test serum itself as the complement source, which may be inconvenient for several reasons: all sera must be collected by standardized procedures to preserve complement activity, sera must be tested at high concentrations to avoid diluting the complement components that will consequently make it difficult to differentiate between stronger responses (as some responses may be more or less off scale), and there is high consumption of serum. Also, the complement activity and

other serum factors may differ from one person to another, and the responses measured may thus not reflect specific antibody responses. In our RB assay we use an external complement source, ensuring identical complement activity among the different serum samples, and the dilution effect is omitted, since a constant amount of complement source is added to all test serum dilutions.

We prefer to measure RB rather than phagocytosis. This late event of phagocytosis is probably more related to bacterial killing than mere ingestion and is therefore more relevant to measure in vaccine response studies. Phagocytosis (ingestion) might also be measured in a similar flow cytometric technique, but then the bacteria have to be labelled with a fluorochrome such as FITC (1). Such surface labelling might modify or disguise relevant antigens, and additional techniques must be introduced to discriminate between internalized and adherent bacteria.

In conclusion, we have shown a very high correlation between RB and anti-OMV IgG antibodies and a somewhat weaker but significant correlation between RB and SBA. It may be beneficial to use a meningococcal vaccine that induces high opsonophagocytic activity rather than high SBA. This will facilitate intracellular destruction of the meningococci that subsequently may reduce intravascular release of hazardous endotoxins seen with prolonged (enhanced) complement activation (4, 21). This may be particularly important if the antimeningococcal activity is not able to stop the invasion of meningococci into the bloodstream at an early stage (with a low bacterial load). These considerations may support the use of a RB assay as an additional screening test in the surveillance of the immune response in vaccine trials.

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