Investigation on the Effect of Immune Selection on Resistance to Bactericidal Antibodies to Group B Meningococci In Vitro

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The induction of resistance by immune selective pressure to bactericidal antibodies from humans immunized with Novartis recombinant meningococcal group B vaccines was assessed. Serum bactericidal antibody titers against selected bacteria were within assay variability through a selection event frequency of 1 in 10^{-5}. No change in antigen expression was observed by Western blotting.

The development of a broadly protective vaccine against group B meningococci (MenB) has been hampered by ineffective MenB capsular polysaccharide vaccines and extensive variability of outer membrane proteins targeted by vesicle-based vaccines (1, 11, 15, 16). We have approached this problem by selecting candidate antigens encoded on the MenB genome that can individually serve as targets for protective antibody responses yet are very distinct from each other (6, 13). The use of diverse, highly conserved antigens could reduce the possibility of immune escape due to vaccine-induced selective pressure (17). However, given the high degree of genetic instability observed for group B strains (5, 7, 8, 12), it is possible that resistance to bactericidal antibodies could be induced by vaccination. To address this question, we have selected bacteria with human serum complement and bactericidal antibodies induced by one of two vaccines containing 25 μg each of three recombinant proteins known to elicit bactericidal responses in mice—NadA as a single polypeptide and factor H-binding protein (fHBP) and GNA2132 fused to carrier proteins as GNA2091-fHBP and GNA2132-GNA1030—either alone (6) or in combination with 50 μg outer membrane vesicles from strain H44/76, with each vaccine in 1.5 mg of aluminum hydroxide per 0.5-ml dose. Sera were collected 1 month after immunization with three doses of vaccine given at 1-month intervals, after a fourth immunization given 4 months after the third dose, or after a fifth immunization given 12 months after the fourth dose. Control sera were obtained before the first immunization and pretested to ensure that they lacked naturally acquired bactericidal antibodies against strains H44/76 and 2996.

The bactericidal assay was performed using human complement as described previously (14). Briefly, frozen stock cultures of bacteria were grown overnight on chocolate agar. The next day (day 1), 10 to 20 colonies were selected, pooled, and grown in Mueller-Hinton broth (Becton-Dickinson) for approximately 2 h to mid-log phase. Bacteria were then diluted to a concentration of 2.0 \times 10^8/ml for use in the assay. Test sera were serially diluted twofold in 96-well plates starting from a 1:2 dilution and then incubated for 60 min with bacteria and 25% human serum complement lacking intrinsic bactericidal activity. Aliquots were spread onto chocolate agar plates and grown overnight. All bacterial cultures were grown at 37°C in 5% CO₂. On day 2, colonies were counted and the 50% titer of each test specimen relative to that of the time zero inoculum was determined. Surviving bacterial colonies from the serum dilution treatment that resulted in 90% killing of bacteria were collected from the agar plate and pooled to prepare a new broth culture on day 2 that was then immediately reasayed in the next cycle. This process was repeated for up to five rounds of selection. We chose to use five rounds of 10-fold reduction

| Strain and human serum sample | Log2 titer (antibody selected) | Log2 titer (control) |
|------------------------------|-------------------------------|---------------------|
| H44/76                        |                               |                     |
| Sample 1                      | 0.62071                       | -0.11984            |
| Sample 2                      | 0.82312                       | 0.10154             |
| Sample 3                      | -0.18057                      | -0.42489            |
| Sample 4                      | -0.79647                      | -0.51281            |
| 2996                          |                               |                     |
| Sample 5                      | 1.04891                       | -1.03747            |
| Sample 6                      | -0.24511                      | 0.10685             |
| Sample 7                      | -0.74250                      | -0.60572            |
| Sample 8                      | 0.07400                       | -0.84800            |

Mean difference of log2 titers 0.07526 -0.41733 -0.57365 -0.37616

Antilog (mean difference of log2 titers) 1.03555 1.33546 1.48828 1.29788

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TABLE 2. Difference in bactericidal titers obtained against strain H44/76 after a fifth cycle of selection

| Human serum sample | Log2 SBA titer vs bacteria passaged in: | Difference of log2 titers |
|--------------------|----------------------------------------|--------------------------|
|                    | Normal serum                           | Immune serum             |                           |
| 9                  | 6.79442                                | 5.61471                  | −1.17971                 |
| 10                 | 6.90689                                | 5.72792                  | −1.17897                 |
| 11                 | 6.95420                                | 5.75489                  | −1.19931                 |
| 12                 | 5.72792                                | 4.58496                  | −1.14296                 |
| 13                 | 6.84549                                | 5.88496                  | −1.26053                 |
| 14                 | 5.95420                                | 4.16993                  | −1.78427                 |
| 15                 | 6.30378                                | 5.08746                  | −1.21632                 |
| 16                 | 8.08215                                | 6.38496                  | −1.49719                 |
| 17                 | 7.05528                                | 5.55459                  | −1.50069                 |
| 18                 | 6.61471                                | 5.28540                  | −1.32931                 |

Mean difference of log2 titers: −1.32892

Antilog (mean difference of log2 titers): 2.51215

a log2, logarithm with base 2; antilog, power function with base 2 (inverse function of log2 function).

FIG. 1. Immune selection by vaccine-induced serum bactericidal antibody and human complement did not affect the level of expression of NadA, fHBP, GNA2132, or PorA compared to the wild-type expression as determined by Western blotting for strains H44/76 (A to C) and 2996 (D to G). Knockout mutants for fihbp, gna2132, and nadA prepared in strains MC58, 8047, and NMB, respectively, were used as negative controls. The H44/76 strain lacks the gene for NadA. In colony counts (overall, a 10,000-fold selection) based on studies of phase variation in MenB in which individual genes underwent phase variation with frequencies of 1 in 10^4 to 1 in 10^5 (9). A pool of surviving colonies was used in each round instead of single colonies being picked posttreatment to optimize the chance of passing a selection variant. Also, the use of pooled colonies is a standard practice when performing bactericidal assays on meningococci (3). The criterion used to assess induction of resistance was a fourfold or greater reduction in titer compared to that of control bacteria. This criterion was selected because the sera are titrated in a twofold serial dilution and a difference of two titer steps is the accepted criterion for a measurable difference in results (4), which reflects the inherent variability of the assay.

The study included two types of planned comparisons. First, we compared the titers of a set of immune serum against...
bacteria that had been selected over four rounds of selection in that same serum to the titers obtained against bacteria treated with control serum. The effect of this selection process using strains H44/76 and 2996 is shown in Table 1. A fourfold or greater reduction in the mean difference in titer was not observed at any of the four selection steps. The mean difference after the fourth cycle of selection was approximately 1.3-fold. Mean differences were calculated using logarithmically transformed titers (logarithm to base 2). Second, we compared the bactericidal titers of 10 immune sera using strain H44/76 after a fifth selection cycle to the results with the control treatment. The mean difference in titers was approximately −2.5-fold (Table 2). Overall, there was a slight trend toward lower titers when bacteria were selected with a serum containing bactericidal antibody.

The effect of immune selection on antigen expression in strains H44/76 and 2996 is shown in Fig. 1. Lysates from wild-type and antibody-selected bacteria after a fifth passage were probed by Western blotting to examine changes in the expression levels of NadA, iHBp, GNA2132, and PorA. Bound proteins were detected using horseradish peroxidase-conjugated rabbit anti-mouse or goat anti-rabbit polyclonal antibody (Dako) as appropriate, followed by treatment with the Opti-4CN substrate kit (Bio-Rad). MenB strains with the genes encoding the relevant antigens deleted, MC58Δhfhp (10), 8047Δgna2132, and NMBΔnadA, were used as negative controls. Immunostaining of antibody-selected bacteria demonstrated antigen levels comparable to those of wild-type bacteria. We conclude from this series of experiments that within the selection conditions used, there did not appear to be a biologically significant effect on the resistance of bacteria to killing by vaccine-induced antibodies or an alteration in the surface expression of vaccine-related antigens.

Serum bactericidal antibody is a well-accepted correlate of protection against meningococcal disease recognized by regulatory agencies around the world, including the FDA and the EMEA (4). Because of the extensive diversity observed in MenB strains for antigens such as the porins and pili (1, 2, 16), it is possible that resistance to bactericidal antibodies might occur via selective pressure from vaccination. While it is difficult to model in vitro the cumulative effects of the general use of a vaccine in a large human population over a sustained period, these studies show that within the expected range of appearance of phase variants, a loss of susceptibility to bactericidal antibodies raised by these MenB vaccine combinations was not observed.

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