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Effect of a booster dose of serogroup B meningococcal vaccine on antibody response to *Neisseria meningitidis* in mice vaccinated with different immunization schedules

Carolina A.C. Gioia [a,b], Adriana B. de Sousa [b], Simone C. Cruz [b], Feliciano C.S. Junior [b], Arnaldo F.B. Andrade [b], Raul M. Sassi [a], Carl E. Frasch [c], Lucimar G. Milagres [b,*]

[a] *Fundação Universidade Federal de Rio Grande, Hospital Universitário, Departamento de Patologial/Microbiologia e Imunologia, Rua Osório S/N. CEP: 96200400. Rio Grande, RS, Brasil*

[b] *Universidade do Estado do Rio de Janeiro, Bl. 28 de Setembro, 87, Fundos 3° andar, Disciplina de Microbiologia e Imunologia, CEP: 20551030, Rio de Janeiro, RJ, Brasil*

[c] *Laboratory of Bacterial Polysaccharides, Center for Biologics Evaluation and Research, FDA, 8800 Rockville Pike, Bethesda, MD 20892, USA*

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Abstract

The generation and maintenance of memory antibody response by different primary immunization schedules with the Cuban-produced outer membrane protein based vaccine was investigated in a murine model. We analyzed the duration of the antibody response (IgG-ELISA and bactericidal titer) and the effect of a booster dose on the antibody response. The IgG avidity index was determined in an attempt to find a marker for memory development. This study also included an analysis of IgG subclasses induced by primary and booster immunization. The specificity of bactericidal antibodies was investigated using local strains of the same serotype/serosubtype (4,7:P1.19,15) as the vaccine strain and mutant strains lacking major outer membrane proteins. A significant recall response was induced by a booster dose given 7 months after a primary series of 2, 3 or 4 doses of vaccine. The primary antibody response showed a positive dose-effect. In contrast, a negative dose-effect was found on the booster bactericidal antibody response. There was a significant increase in IgG1 levels after the fourth and booster doses. Three doses of vaccine were required to induce a significant increase in IgG avidity. Two injections of vaccine induced a significant antibody response to PorA protein, while 4 injections induced a larger range of specificities.
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1. Introduction

In the last decades several MenB (*Neisseria meningitidis* B) vaccines based on lipo-oligosaccharide (LOS)-depleted outer membrane proteins (OMPs) have been developed and tested in humans. However, the efficacy and/or effectiveness of two doses of these vaccines have shown important limitations [1]. The Cuban-produced OMP (B:4,7:P1.19,15 strain) based vaccine (VA-MENGOC-BC®) was previously used to immunize millions of children in São Paulo and Rio de Janeiro, (Brazil). Despite a satisfactory vaccine efficacy (74%) in children aged over 48 months, younger children were poorly protected [2,3].

The factors related to the limited performance of VA-MENGOC-BC® vaccine in Brazil are unknown.

* Corresponding author. Tel.: +55 21 2587 6380; fax: +55 21 2587 6476.

E-mail address: lucimar@uerj.br (L.G. Milagres).

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Previous study showed PorA (P1.19,15) as an important target antigen recognized by the bactericidal antibodies from vaccines [4]. However, investigations did not show any point mutations of VRs of the *por*A gene in P1.19,15 strains recovered from vaccine failures [5].

The immunogenicity of several MenB vaccines has recently been tested using a primary immunization schedule of 3 or 4 doses [6–9]. The administration of 3 doses of the Norwegian MenB vaccine in teenagers induced a higher and long-lasting (at least 1 year) antibody response when compared with 2 doses [6]. A primary series of 3 doses of the Cuban MenB vaccine has also induced a higher antibody response in children when compared with only 2 doses [9].

A combination of pre-existing antibodies and rapid secondary immune response is likely important in protection against the meningococcal disease. A significant recall response against meningococci was reported in about 48% of children previously vaccinated with 3 doses of a recombinant hexavalent PorA MenB vaccine [7]. Previous reports have shown that meningococcal disease patients previously immunized with MenB vaccines mounted a higher bactericidal antibody response to B meningococci than non-immunized patients [10,11]. This may well reflect priming for immunologic memory by these vaccines.

There is a great need for laboratory correlates of protection against meningococcal disease. Antibody avidity index has been used as a surrogate of development of immunological memory upon vaccination against *Haemophilus influenzae* [12]. A recent report showed a significant increase in avidity index of PorA specific antibodies during the course of vaccination and after a booster dose of a recombinant monovalent PorA MenB vaccine. A high antibody avidity index was associated with high bactericidal activity [13].

In this study, we used an animal model to investigate the effect of different primary immunization schedules with the MenB Cuban vaccine on the magnitude and persistence of antibody response (IgG and bactericidal antibodies), and on development of immunological memory given by the antibody recall response induced by a booster dose of vaccine.

2. Materials and methods

2.1. Serogroup B meningococcal strains

The Cuban vaccine strain (Cu385/83) of serotype/serosubtype/immunotype 4,7:P1.19,15:L3,7,9 was used to measure the kinetics of IgG and bactericidal antibody titers of immunized mice. Local strains, N244/96 (B:4,7:P1.19,15:L(1),2,(3,7,9)) and N43/90 (B:4,7:P1.19,15:L3,7,9,(8)), expressing the same sero/serosubtype as the vaccine strain were used in the bactericidal assay with a set of sera.

Two mutant strains derived from strain H355/75 (B:15:P1.19,15:L3,7,9,8) were used. Strain H355/75 PorA$^-$ was kindly provided by Dr. Gerardo Guillen, Centro de Ingeniería Genética y Biotecnología (Havana, Cuba). Strain H355/75 Opa$^-$ (class 5 OMP) was obtained as described previously [14] using monoclonal antibody (mAb) 3DH3-F5GE (BioManguinhos, Fiocruz) that recognizes epitope 5 of the Opa protein and rabbit serum as the complement source. The absence of PorA and Opa proteins was investigated using SDS–PAGE followed by silver stain analysis and by the lack of reactivity with a panel of subtype and class 5 mAbs.

2.2. Vaccine and immunization of mice

The Cuban serogroup B vaccine (VA-MENGOC-BC®) was kindly provided by Laboratório ENILA and GlaxoSmithKline, Rio de Janeiro, Brazil.

Five- to 6-week old female Swiss mice in groups of 12 were immunized with 2 (BC2 group), 3 (BC3 group) or 4 (BC4 group) intramuscular injections of vaccine, 2 to 3 weeks apart. Each vaccine injection (100 μl) contained 2 μg of OMPs, 2 μg of C polysaccharide, 400 μg of $Al(OH)_3$ and 0.01% of thimerosal as preservative [15]. Mice were bled before each injection and at 14 days, 2, 5 and 7 months after the last injection. Approximately, 7 months after the last dose, mice in each study group received a booster injection of the vaccine. Blood samples were collected 14 days after the booster dose. The serum samples were stored at −20 °C.

2.3. ELISA and immunoblot assays

Outer membrane vesicles (OMVs) from Cu385/83 strain were used as the coating antigen. ELISA and Immunoblot (IgG) analysis were performed as previously described [16] with a peroxidase-conjugated anti-mouse IgG (KPL), IgG1, IgG2a, IgG2b, IgG3 (all IgG isotypes were from Zymed) and IgM (Sigma). As an internal antibody standard for IgG determination, a twofold dilution series of a pool of positive post-vaccination sera, assigned 1000 U ml^{-1}, was used in all ELISA experiments. The mean value of the observed optical density was transformed to arbitrary units per milliliter by a sigmoidal curve (four-parameter logistic log transformation) calculated from the values of the standard serum [17].

IgG subclass concentrations were expressed as the dilution of serum giving an optical density of 490 nm ($OD_{490\ nm}$) of 0.5 (located in the linear region of the curve).

2.4. Bactericidal assay

Serum bactericidal antibodies were measured as previously described [4] with some modifications. Briefly, the final reaction mixture contained 25 μl of diluted test serum previously heat inactivated at 56 °C for 30 min, 12.5 μl of guinea pig serum that lacked detectable intrinsic bactericidal activity and diluted at 1:2, and 12.5 μl of log phase meningococci (about 5×10^3 cfu ml^{-1}) grown on Tryptic Soy Broth (Difco) solidified with 1.5% (w/v) Noble agar (Difco) and containing 1% (v/v) horse serum. The bactericidal reaction was carried out at 37 °C for 30 min. The bactericidal titer was defined as the reciprocal of the serum dilution (before addition of complement and bacteria) causing $\geqslant$50% killing and recorded as the $\log_2$ titer. A value of 1 was assigned to each titer of <2; thus, $\log_2^1 = 0$. The positive control for each assay consisted of a pool of post-vaccination mouse serum with previously determined bactericidal titer. The negative control consisted of the complement source in the absence of test serum.

2.5. Antibody avidity assay

Antibody avidity was measured by an elution ELISA using the chaotrope, thiocyanate, as described previously [18]. Briefly, sera were diluted in buffer containing 10 nM PBS, 0.05% Tween 20 and 1% BSA to a final concentration to obtain an optical density of ~1.0 (linear region of the dilution curve) and then incubated on an antigen-coated plate at 37 °C for 2 h. Antigen-coated plates were prepared using 4 μg ml^{-1} OMV from Cu385 strain in Tris–HCl buffer, pH 8.0. The plates were then washed and sodium thiocyanate (Sigma) diluted in PBS at different concentrations, ranging from 0 to 5 M, was added to the wells. After 15-min incubation at room temperature, the plates were washed and antibody detected with peroxidase-conjugated anti-mouse IgG incubated for 90 min. The assay was developed using *o*-phenylenediamine (Sigma) in 0.05 M phosphate citrate buffer (pH 5.0) and the reaction stopped after 30 min with 4 N sulphuric acid. Results were expressed as an avidity index corresponding to the molar concentration of sodium thiocyanate required to produce a 50% reduction in absorbance.

2.6. Statistical analyses

The ELISA and bactericidal results were transformed to logarithmic values to calculate the geometric and arithmetic means, respectively. The significance levels of differences between groups were examined by Student's *t*-tests or Maan–Whitney test. $P < 0.05$ was taken as significant.

3. Results

3.1. Kinetics of IgG antibody response

Fig. 1 shows the geometric mean (GM) of IgG anti-B:4:P1.15 OMVs (strain Cu385/83) of mice from vaccine groups BC2, BC3 and BC4. A second immunization markedly boosted antibody levels of animals from all vaccine groups (GM levels varied from 301 to 438 U ml^{-1}). Due to the large individual variations in the kinetics of antibody development, we grouped all the animals that received 2 and 3 doses from each vaccine group to calculate the significance of the IgG response after different doses of vaccine. There was a

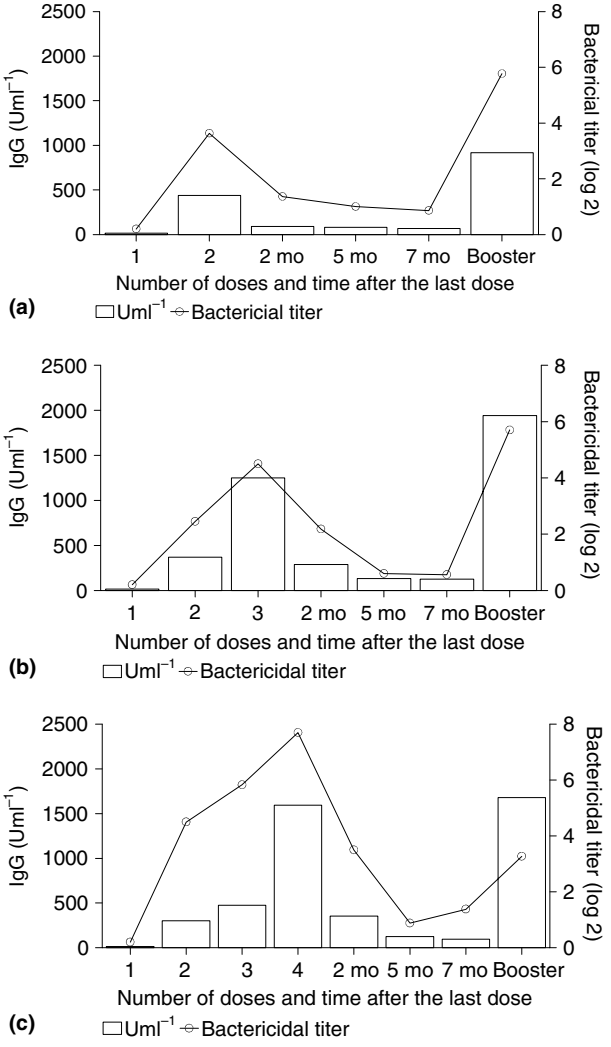

Fig. 1. Geometric mean of ELISA IgG levels (left *Y* axes, U ml^{-1}) and mean (log2) of bactericidal titer (right *Y* axes) against Cu385 strain (B:4,7:P1.19,15) in individuals from groups BC2 ((a), n = 10–11), BC3 ((b), n = 9–11) and BC4 ((c), n = 11–12). The *X* axes shows the number of doses and time after the last dose. Blood samples were collected 14–21 days after each dose and at 2, 5 and 7 months after the last dose. Booster dose was given about 7 months after the last dose and blood samples were collected 14 days after booster.

great ($P = 0.008$) increase of IgG levels after 3 doses (GM of 714 U ml^{-1}) compared with 2 doses (GM of 370 U ml^{-1}) and also after the fourth dose (GM of 1594 U ml^{-1}) compared with the third dose ($P = 0.02$).

Two months after the last dose, we detected a significant decrease in antibody levels in all vaccine groups (mean decrease of 4.5-fold). The IgG levels of group BC2, two months after the second dose was lower than that observed in the other groups ($P < 0.05$). By 5 months after the last dose, there was no significant difference in antibody levels between the groups.

The booster dose given 7 months after the last dose induced a significant increase in IgG levels (mean increase of 14-fold) independent of the primary immunization schedule. Remarkably, the booster response of BC2 (GM of 916 U ml^{-1}) and BC3 (GM of 1942 U ml^{-1}) groups was higher ($P < 0.05$) than the primary respective IgG response (GM of 370 and 714 U ml^{-1}, respectively). In contrast, the IgG booster response (GM of 1681 U ml^{-1}) and the primary antibody response (GM of 1594 U ml^{-1}) of group BC4 were similar ($P > 0.05$).

3.2. Kinetics of bactericidal antibody response

As shown in Fig. 1, a significant antibody response was observed after the second injection of vaccine for all vaccine groups (log2 mean titer [LMT] of 3.5). As for IgG-ELISA, we detected a great individual variation in bactericidal antibody titers. After grouping all the animals that received 2 ($n = 35$) and 3 ($n = 21$) doses, we detected a significant increase ($P = 0.01$) in bactericidal antibodies after 3 doses (LMT of 5.3) compared with 2 doses (LMT of 3.5). In animals from group BC4 the fourth dose induced a significantly higher antibody response (LMT of 7.7, $n = 12$) than the third dose (LMT of 5.8). Therefore, there was a dose related increase in bactericidal antibody response after the primary immunization. After 2 and 3 doses, 34% and 9.5% of animals, respectively, had bactericidal titers lower than 3 (log2). After the fourth dose, there was no individual with bactericidal response inferior to 3 (data not shown).

A significant decrease (mean decrease of 2.3-fold) in antibody levels was observed 2 months after the last dose for all vaccine groups (Fig. 1(a)–(c)). However, higher bactericidal antibody levels were detected at that time in animals from group BC4 (LMT of 3.5) compared with group BC3 (LMT of 2.2) ($P = 0.057$) or group BC2 (LMT of 1.4) ($P < 0.05$). Five months after the last dose, there was no significant difference in bactericidal antibody levels between groups (LMT of 0.83).

The booster dose induced a significant increase in bactericidal antibodies compared with pre-booster (7 months) response in all vaccine groups (increment of 6.7, 10.2 and 2.4-fold for groups BC2, BC3 and BC4, respectively). The booster response was higher than the primary response detected in mice from groups BC2 (increment of 1.6-fold) and BC3 (increment of 1.3-fold). However, animals that had received 4 doses (group BC4) responded less ($P < 0.05$) to the booster dose compared with the primary antibody response (decrease of 2.4-fold). The results reported above for groups BC2 and BC4 were representative of 2 independent experiments.

Taken together the IgG and bactericidal antibody response to Cu385 strain indicated a dose-related increase in antibody response. Despite the rapid decrease (2 months) in antibody levels induced by the primary immunization schedules, a significant booster response was induced 7 months after the last dose. However, as indicated by the bactericidal antibody data, there were differences in the functional booster antibody responses of mice primed with 4 injections of vaccine.

3.3. Bactericidal antibody response against different P1.19,15 strains and mutant strains

Fig. 2 shows the bactericidal titers of selected sera (based on the available volume) from groups BC2 and BC4 against H355 strain and its variants (PorA$^-$ and Opa$^-$ strains). Sera collected after 2 doses of vaccine had significantly lower antibody titers to H355 PorA$^-$ strain (LMT of $\leqslant$1.6) compared with the parent strain (LMT of 4.7) (Fig. 2(a)). In contrast, H355 Opa$^-$ strain was more susceptible ($P < 0.05$) to lysis by antibodies (LMT of 7.5) than the parent strain. In comparison, sera from individuals that received 4 doses of vaccine (group BC4) showed an increased ability to kill the PorA$^-$ strain (Fig. 2(b)). There was no significant difference between bactericidal antibody titers against H355 (LMT of 7.6) and its PorA$^-$ mutant (LMT of 5.2) detected in sera from group BC4. Again, the Opa$^-$ strain was more susceptible to lysis (LMT of 9.7). The reasons of why H355 Opa$^-$ strain presented higher susceptibility to killing by bactericidal antibodies when compared to strains H355 and H355 PorA$^-$ are unknown. A comparative analysis of expression of L3,7,9 and L8 immunotypes by the parent strain (H355) and its variants (PorA$^-$ and Opa$^-$) could add important information since expression of L8 immunotype is correlated with increased sensitivity to serum bactericidal activity [19]. In summary, after the primary immunization, group BC4 responded with a significantly higher response to strains H355 and its Opa$^-$ mutant compared with group BC2. Higher ($P = 0.059$) antibody levels to the H355 PorA$^-$ mutant were also detected in group BC4 compared with group BC2.

Fig. 2 also shows the bactericidal antibody response to H355 strain and its variants after the booster dose. Individuals from group BC2 showed a significant increase in bactericidal antibody titers against strains H355 and its Opa$^-$ mutant, but not to the PorA$^-$ mutant compared with the titers induced by 2 doses. The

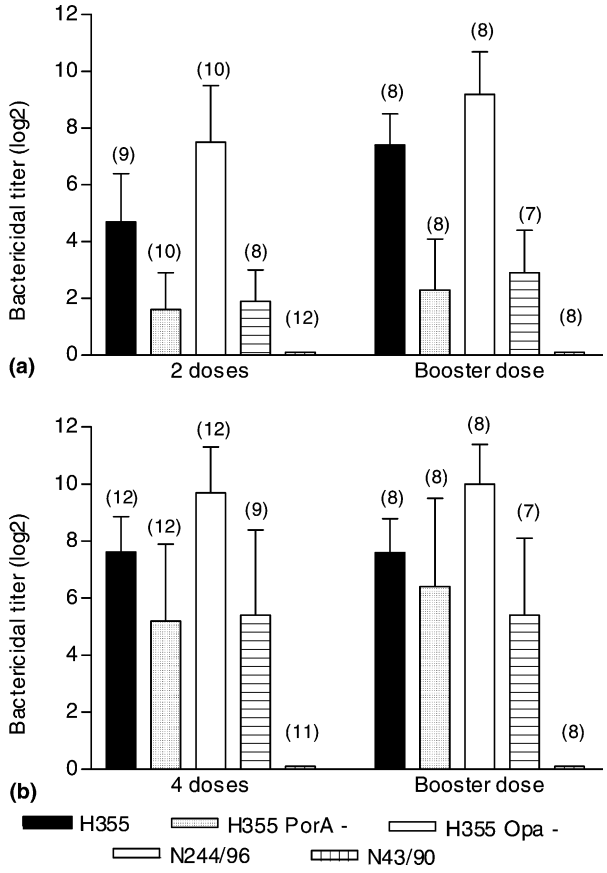

Fig. 2. Bactericidal antibody levels (mean log2) of sera from mice of vaccine group BC2 (Fig. 1(a)) and group BC4 (Fig. 1(b)) against local strains (N244/96 and N43/90) of the same serotype/serosubtype (4,7:P1.19,15) as the vaccine strain and mutant strains lacking PorA or Opa protein. Numbers above the bars indicate the number of sera tested. Error bars show the standard deviation. Blood samples were collected 14–21 days after the primary immunization. A booster dose was given about 7 months after the last dose and blood samples were collected 14 days after booster.

booster dose had no further effect on peak of bactericidal titers in group BC4. There were no significant differences in the magnitude of bactericidal antibody response to strains H355 and its Opa$^-$ mutant between groups BC2 and BC4 after the booster dose.

Concerning the different P1.19,15 strains used in this study, we observed a lower ($P < 0.05$) bactericidal antibody response to N244/96 strain compared with strains H355 and Cu385/83 after the second (LMT of 1.9, 4.4 and 3.2, respectively) and fourth (LMT of 5.4, 7.9 and 8.1, respectively) dose of vaccine. Group BC2 responded to the booster dose with lower ($P < 0.05$) bactericidal antibody titers to strain N244/96 (LMT of 2.9) when compared with strain Cu385 (LMT of 5.7). However, for group BC4 there was no significant difference between the bactericidal antibody response to strains N244/96 and Cu385 after the booster dose.

No sera from group BC2 or BC4 collected after the primary or booster vaccination were bactericidal for strain N43/90. This strain naturally expresses low amounts of PorA protein, detected by SDS–PAGE and Western-blot using P1.15 mAb [4]. This may explain the resistance of strain N43/90 to bactericidal antibodies shown in this study.

We conclude from this experiment on selected sera that 2 doses of vaccine induced a significant antibody response to PorA protein while 4 doses also induced antibodies with other specificities. Importantly, the three P1.19,15 (H355, N244/96 and N43/90) strains used in this study showed different susceptibilities to antibody-induced lysis.

3.4. IgG avidity

In an attempt to investigate a marker for memory antibody response, we determined the IgG avidity index (AI) 14 days after 1, 2, 3 and 4 doses of vaccine in animals from group BC4. The IgG AI in serum samples obtained before booster and 14 days after booster was measured in all vaccine groups. As shown in Table 1, the IgG AI increased after each dose and over time.

The IgG AI did not differ significantly between groups at any time point analyzed. There were no differences between the AI induced using 1 dose (AI = 0.80)

Table 1
Geometric mean (95% confidence interval) avidity index of IgG anti-OMVs of *N. meningitidis* B:4:P1.15 (strain Cu385/83) after primary immunization of mice from group BC4 (1, 2, 3 and 4 doses) and before and after boosting of group BC4, group BC3 and group BC2 with Men B vaccine

Doses	*n*	Group BC4	Group BC3	Group BC2
1	13	0.80 (0.68–0.92)		
2	10	0.96 (0.69–1.23)		
3	9	1.37 (0.94–1.80)[c]		
4	9	1.72 (1.37–2.07)[d]		
7 months	9[a]	2.31 (1.73–2.89)	2.02 (1.59–2.45)	2.33 (1.80–2.86)
Booster	10, 5, 11[b]	2.37 (2.06–2.68)	2.03 (1.51–2.55)	2.30 (2.08–2.52)

[a] $n = 9$ for each study group.
[b] $n = 10$, 5 and 11 for groups BC2, BC3 and BC4, respectively.
[c] $P < 0.05$ compared to 1 dose.
[d] $P < 0.05$ compared to 1 and 2 doses.

and 2 doses (AI = 0.96) or 2 and 3 doses (AI = 1.37) of vaccine. The IgG AI detected after the third (AI = 1.37) and fourth (AI = 1.72) dose was higher ($P < 0.05$) than the AI detected after the first dose (AI = 0.80). A significant difference was also found when we compared the AI induced by 2 (AI = 0.96) and 4 doses (AI = 1.72). In contrast, there was no important ($P > 0.05$) difference between the AI determined before (AI = 2.02–2.33) and after the booster dose (AI = 2.03–2.37) for each study group. However, for each study group, the AI detected after booster dose was higher than the AI detected after the primary immunization schedule ($P < 0.0001$ for BC2 group, $P = 0.001$ for BC3 group and $P = 0.01$ for BC4 group).

There was no correlation between AI and bactericidal activity after 2, 3 or 4 doses of vaccine ($r = 0.06$, -0.04 and 0.1, respectively, $P > 0.05$).

3.5. IgG subclasses

The profile of IgG subclasses was investigated to assess its correlation with bactericidal activity and the number of injections of vaccine. IgG2a, IgG3 or IgM were not detected in sera from any study group at any time point (data not shown).

The results shown in Fig. 3 demonstrated that for both groups studied there was a predominance ($P < 0.05$) of IgG2b over IgG1 after the primary immunization. There was no significant difference in IgG2b levels after 2 (GM titer of 22452) or 4 (GM titer of 28225) doses. A positive correlation ($r = 0.89$, $P = 0.0001$) was found between IgG2b and bactericidal titers induced by 4 doses of vaccine.

For group BC4 (Fig. 3(b)), the IgG2b levels were higher ($P < 0.05$) after the second (IgG2b:IgG1 = 6.5) and third (IgG2b:IgG1 = 5.5) dose compared with IgG1 levels. The fourth injection of vaccine induced a significant increase in IgG1 levels. In consequence, the proportion of IgG2b:IgG1 decreased to 2.3.

For both groups, the booster dose induced a significant increase in IgG1 levels compared with the levels detected before the booster or 14 days after the primary immunization schedule. The levels of IgG2b also increased after the booster dose but in contrast to IgG1 levels it did not change (group BC4) or decreased (group BC2) when compared with the last dose of the primary immunization schedule.

We concluded from this experiment that the increase in the number of vaccine injections favours development of IgG1 antibodies. This was observed after the fourth dose and also after the booster dose given to both study groups. Future studies addressing the cytokine profile and expression of CD40L after immunization may add important information to explain the IgG subclasses profile induced by different immunization schedules.

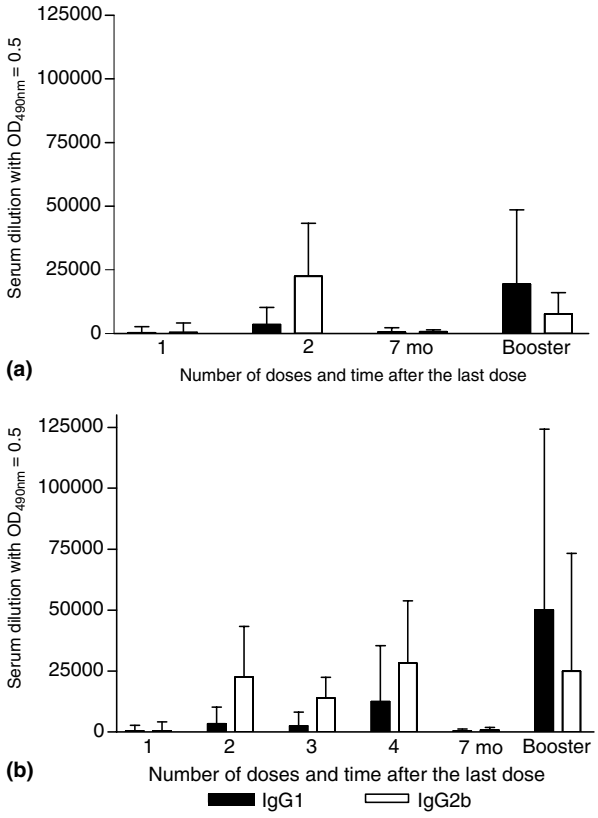

Fig. 3. Geometric mean of IgG1 and IgG2b levels of mice from group BC2, ((a), $n = 10$–11) or group BC4 ((b), $n = 9$–12) specific to Cu385 strain (B:4,7:P1.19,15). Error bars show the standard deviation. Sera were collected 14–21 days after each dose and 7 months after the last dose of vaccine. A booster dose was then injected in animals and IgG subclasses were measured 14 days after the booster injection.

4. Discussion

Significant efforts are being made to develop new and effective meningococcal vaccine strategies [20]. Considerable evidence indicates that PorA antibodies play an important role in immunity to *N. meningitidis* B [4,10,21]. Recently, important studies of immunological memory induced by MenB vaccines have been reported [6–8,13].

This study was primarily designed to investigate the generation and maintenance of memory antibody response comparing different primary immunization schedules in a murine model. We showed that a significant antibody recall response was induced by a booster dose given 7 months after a primary series of 2, 3 or 4 doses of vaccine. In contrast to IgG-ELISA data, the bactericidal antibody response indicated a difference in the recall response of mice primed with 4 injections of vaccine. The data, unexpectedly, demonstrated a negative dose-effect on booster bactericidal antibody response, in that individuals from group BC4 responded less well to the booster dose compared with the primary series (four doses). There are several possible explana-

tions for our finding of a negative dose-effect on booster bactericidal response but not on IgG recall response, acting alone or in concert. First, larger number of vaccine injections (total of 5) could induce blocking IgG. Though administration of 3 doses of the Norwegian MenB vaccine has not induced anti-class 4 blocking antibodies in humans, mouse monoclonal antibodies to class 4 OMP can block bactericidal killing by anti-PorA mAb [21,22]. In fact, despite the less potent booster bactericidal antibody response of group BC4, the recall IgG response was similar to the primary response. In addition, immunoblot studies showed a marked IgG binding to class 4 OMP after the third, fourth and the booster dose of vaccine (data not shown). It should also be noted that there was a significant increase in IgG1 after the fourth and booster doses of vaccine. Therefore, future studies should address the function and specificities of purified IgG1 antibodies induced by vaccination. Second, the fine specificity of antibodies induced by the booster dose may differ from that induced by the primary immunization schedule. These may include epitopes that do not induce bactericidal antibodies. Moreover, for group BC4 we observed a negative correlation ($r = -0.54$) between IgG levels and bactericidal titers after the booster dose. Finally, the IgG booster response could also have a biological function other than bactericidal activity such as being opsonic. This is being investigated currently.

Our data showed a need for 3 doses of vaccine to induce a significant increase in avidity of specific IgG during the primary series. Thereafter, the AI increased over time and was similar before and after the booster dose for all study groups. These findings may indicate an already matured antibody response to the protein antigens after 3 doses of vaccine. Notably, the maturation of IgG avidity over time was similar for animals that received 2 or 4 doses of vaccine. In contrast, an increase in IgG AI after the booster dose in infants who had been immunized with 3 or 4 doses of a recombinant hexavalent PorA vaccine have been reported [8]. In another human study, an increase in IgG AI was found after the booster dose in toddlers previously vaccinated 2 or 3 times with a monovalent PorA vaccine. In the later study, the IgG AI was assessed using a monovalent OMV suspension lacking major antigens like class 3 and class 4 OMPs [13]. Remarkably, both hexavalent and monovalent PorA vaccines did not contain class 3 and class 4 OMPs. Taken together, differences in vaccine and ELISA antigens used in these human studies may have contributed to the different results reported in our study using an animal model and a non-recombinant OMP vaccine.

The present study showed that four doses of vaccine were important to induce a large range of bactericidal antibody specificities. According to our results, a PorA$^-$ strain was susceptible to vaccine induced antibodies compared with its parent strain after four doses but not after two doses. These results are in agreement with a previous report that showed the importance of a third dose to increase the magnitude of antibody response and to induce cross-reactivity antibodies [6,21]. Nonetheless, our study showed a great variability in susceptibility of meningococcal strains to the biological action of antibodies even after 4 doses of vaccine. This was observed by the bactericidal killing resistance of N43/90 strain that expresses a reduced amount of PorA protein. The reasons for the lower susceptibility of strain N244/96 to killing by bactericidal antibodies when compared with strains Cu385 and H355 are unknown. The influence of LOS immunotype of strain N244/96 [L(1),2,(3,7,9)] should be further investigated since it differs from LOS immunotype of strain Cu385 (L3,7,9) and H355 (L3,7,9,8). This strain variation should be kept in mind when bactericidal antibody titers are used as tools to predict effectiveness of new vaccines.

In summary, based on the booster antibody response our results indicated that the use of 2, 3 or 4 injections of an OMP vaccine induced long-lasting immune memory. Interestingly, in terms of development of serological (IgG levels, bactericidal activity and IgG avidity) memory, 2 doses of vaccine had a similar or better effect compared with 4 doses of vaccine. In contrast, the primary antibody response showed a positive dose-effect. Further studies should focus on the frequency of circulating antigen-specific memory B cells since these cells and plasma cells may be independently controlled forms of immunological memory [23]. Accordingly, an indirect correlation was found between the number of previous boosters and the magnitude of specific B-cell expansion after booster immunization of humans with diphtheria toxoid [23]. These results indicated that similar studies would be useful in humans since 2 doses of the Cuban vaccine have not induced good protection in children under 4 years of age [2]. A balanced consideration of the number of doses of vaccine is needed, particularly at a time when new bacterial conjugate vaccines are being licensed and may need to be incorporated into an already complex infant immunization schedule.

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