Analysis of the Ontogeny of the Murine Humoral Response to Neisseria meningitidis B Capsular Polysaccharide Reveals Levels of Complexity Relevant to Vaccine Development

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Although purified capsular polysaccharide of Neisseria meningitidis group B (CpsB) is not immunogenic at any age, CpsB on the bacterial surface elicits antibody responses late in ontogeny. Therefore, a detailed analysis of the ontogeny of the murine anti-CpsB response to N. meningitidis could determine key parameters regarding the poor immunogenicity of CpsB. The effects of bacterial dose, hyperimmunization, age, and sex on the induction of primary and secondary anti-CpsB immunoglobulin isotype profiles were studied. It was demonstrated that the timing and repetition of immunization and of the bacterial dose have a marked differential effect on the primary induction of anti-CpsB immunoglobulin isotypes and on the ability to induce anti-CpsB antibody responses after subsequent rechallenge. It is noteworthy that the ontogeny of the response is related to the appearance of natural anti-CpsB antibodies, but this is not associated with the presence of CpsB cross-reactive antigens in the microflora.

Neisseria meningitidis group B is the major cause of meningococcal disease in developed countries, occurring in >50% of all cases in children <5 years old [1]. Immunity is typically conferred by antibody to capsular polysaccharides (Cps). Purified Cps are poor immunogens in infants and young children, resulting in their heightened susceptibility to the invasive infections caused by encapsulated bacteria. Similarly, the maturation of the humoral response to polysaccharides in the mouse is delayed and does not reach full development until 4 weeks of age or even much later [2, 3]. The reasons for the delay are not well understood but seem to constitute a complex phenomenon partly dependent on Cps structure.

The Cps of N. meningitidis group B (CpsB), a homopolymer of α(2→8)-linked sialic acid residues, are inefficient as an immunogen (even in adults) and when complexed to protein carriers [4–6]. This has hampered the development of an effective vaccine against this meningococcal group. In contrast, the purified Cps of N. meningitidis group C (CpsC), a homopolymer of α(2→9)-linked sialic acid, are immunogenic [2]. However, despite poor CpsB immunogenicity, circulating anti-CpsB antibodies are detectable in nonvaccinated humans and animals [4, 5, 7]. They can be detected in >80% of serum specimens from adults [5, 7]. These natural antibodies have been assumed to be induced by host-carried nonpathogenic bacteria expressing cross-reactive Cps [1, 8–10], Escherichia coli K1 could play a major role in inducing natural anti-CpsB antibodies, because its Cps (CpsK1) is structurally and immunologically indistinguishable from CpsB [9]. Moreover, E. coli K1 is carried by 20%–30% of healthy humans and colonizes the intestinal tract of vertebrates [11, 12].

The expression of long chains of α(2→8)-linked sialic acid antigenically similar to CpsB on vertebrate tissues [13] probably causes the poor immunogenicity of CpsB, because of the induction of tolerance. Polysialic acids are widely expressed on fetal tissues, even though their levels of distribution and expression diminish during the perinatal period [13, 14]. Nevertheless polysialic acid expression continues in the adult, albeit restricted to neural areas capable of plasticity [15–17] and to extraneural cell lineages that express polysialic acids at particular stages of differentiation or functional activity [18, 19]. Thus, the transition from widespread to limited expression of the host polysialic acid occurs during a critical period in the ontogeny of the responses to Cps.

As we reported earlier, CpsB differs from CpsC in being a strictly thymus-independent antigen when presented on the bacterial surface [20]. Herein, we present a detailed analysis of the murine developmental anti-CpsB response to viable N. meningitidis. We studied the effects of bacterial dose, hyperimmunization, and the age and sex of the mouse on the induction of primary and secondary IgM and IgG isotype anti-CpsB antibody profiles during the development of the response. Since one of the current approaches for CpsB-based vaccine development is the use of conjugates of the outer membrane of N. meningitidis or some of its components with the CpsB [5, 6, 21] and since
these vaccines need to be effective earlier in infancy, these studies could help to clarify key parameters relevant to vaccine development.

Materials and Methods

Mice. For study, we used BALB/cOla mice from our own colony at the Centro Nacional de Microbiología (Madrid).

Bacterial strains and Cps. Meningococcal strains were from the culture collection of the National Reference Center for Neisseria. Outer membrane proteins (OMPs) extracted with lithium chloride–sodium acetate from strain B16B6 and CpsB, from CpsC, and from Cps29E from strains B16B6 (B:2a: P1.2:L2{3}), 8148 (C:2b:P1.2), and MABS79 (29E) were purified and characterized, as described elsewhere [20]. The same procedures were used to purify non–O-acetylated CpsK1 from the E. coli strain F11119-41 (O16;K1:H−), which is structurally identical to CpsB [9]. For mouse studies, meningococcal cultures grown on Columbia blood agar for 16 h at 37°C were suspended in PBS to the required density, as estimated by measurement of opacity (Hitachi Science Systems spectrophotometer).

Monoclonal antibodies (MAbs) and antiserum. The production of a polyvalent ascitic fluid against N. meningitidis group B and the 5 IgM (MGB11, MGB12, MGB13, MGB14, and MGB15) and 2 IgG2b (MGB9 and MGB16) murine anti-CpsB MAbs used as standards in the quantitative ELISA have been described elsewhere [22]. The IgG2b and IgM MAbs were purified from culture supernatants by protein-A affinity chromatography and by precipitation with polyethylene glycol 6000, respectively [23]. The biotinylated goat polyclonal antibodies specific for mouse IgM, IgA, IgG, IgG1, IgG2a, IgG2b, and IgG3 and goat anti–mouse IgG(H+L) conjugated to horseradish peroxidase were purchased from Southern Biotechnology. Before these reagents were used, their specificity was confirmed with MAbs of all isotypes purified from culture supernatants.

Immunization protocols. Live N. meningitidis group B strain B16B6 was used for all immunizations. Groups of 20 BALB/c mice (0, 1, 3, 5, 7, 9, 11, and 14 weeks of age) were immunized intraperitoneally with 2 × 10⁸ cfu of N. meningitidis group B. Two weeks later, 10 mice from each age group were administered booster immunizations with either the same dose or 10¹⁰ cfu. Blood samples were obtained from the mice 1 day before the primary immunization and 5–6 days after every immunization, a time by which the anti-CpsB antibody responses had reached maximum levels [20]. To study the influence of hyperimmunization on the development of the anti-CpsB response, we immunized 4-, 6-, and 7-week-old mice twice a week for 4 weeks with 2 × 10⁶ cfu of CpsB. Blood samples were obtained weekly during this schedule and again every 2–3 days after the last immunization. For comparative purposes, age-matched mice were immunized with N. meningitidis group C strain 8148 according to the same schedule.

Search for bacteria expressing Cps cross-reactive with CpsB. The flora of our mouse colony were examined at the onset of these experimental procedures and for ≥6 months afterwards. Groups of 10 mice at the ages of 2 (nursing), 4 (weaned), 5–6 (puberty), and 10 (adult) weeks were used for each examination. Nasopharyngeal, rectal, and vaginal swab samples; ileal, cecal, and urethral homog- enates; fecal emulsions; and urine from these mice were sampled and diluted with warmed tryptic soy broth. Aliquots were plated on Columbia blood, Thayer-Martin, MacConkey, and tryptic soy broth agar media and were incubated at 37°C in a humid 5% CO₂ atmosphere.

After 24 or 48 h of culture, sterile nitrocellulose disks (0.45 μm) were placed on the plate surface and were carefully pressed to ensure complete contact. The disks were removed from the agar plates, dried, and washed to remove the excess bacteria. The washing cycles were done 3 times in PBS for 5 min. Two replicate imprints were obtained for each plate before the cultures were returned to the incubator for further picking of positive colonies. A suspension of N. meningitidis B16B6 strain was dotted on all disks as a positive control. The disks were blocked with 5% skim milk in PBS and washed, and the replicates were incubated overnight at 4°C with a 1:500 dilution of the polyclonal ascitic fluid against N. meningitidis B or a 1:5000 dilution of the MGB12 anti-CpsB MAb ascites diluted in PBS containing 0.05% Tween-20 and 1% bovine serum albumin. MGB12 bound both O-acetylated and non–O-acetylated forms of E. coli CpsK1 [24]. Washed disks were incubated for 2 h with anti–mouse IgG(H+L)–horseradish peroxidase conjugate. After being washed, the disks were stained with a mixture of the chromogenic substrates 3,3′-diaminobenzidine tetrahydrochloride and 4-chloro-1-naphtol [24].

Cultures from digestive tract samples were also tested for E. coli O8 antigen. Disks were treated as described above, except for the use of rabbit antiserum specific for the antigen O8 as primary antibody and anti–rabbit IgG(H+L) as secondary antibody.

ELISA procedures and data analysis. The ELISA protocols and the method for quantitating anti-Cps antibodies have been described in detail elsewhere [20, 22]. Immunoplates presensitized with poly-L-lysine were coated overnight at 4°C with 1 μg/well of CpsB or CpsC in PBS and then were blocked with skim milk. Wells coated with Cps29E, a meningococcal Cps antigenically unrelated to CpsB or CpsC, were used as antigen blanks at each serum sample dilution. In these wells, the IgM-nonspecific binding to poly-L-lysine, which was used to improve the Cps coating, was reduced to a level similar to that in the wells coated with CpsB or CpsC. Furthermore, natural antibodies specific for Cps29-E were undetectable (data not shown). These blanks excluded polyelectrolyte antibodies from natural anti-CpsB antibody determinations. Two-fold serially diluted mouse serum was dispensed in blank and CpsB- or CpsC-coated wells of 6 replicate plates (1 plate for each isotype) and was incubated overnight at 4°C. Since IgM is the major isotype in the anti-CpsB antibody responses and high concentrations of anti-CpsB IgM inhibit IgG binding to CpsB [22], for the IgG determinations the serum was incubated in 0.1 M 2-mercaptoethanol for 15 min at 20°C before being diluted and dispensed into the wells. The antibody bound to the antigen was detected by incubation with the corresponding biotinylated polyclonal antimmunoglobulin isotype for 1.5 h at 37°C and then with streptavidin peroxidase conjugate for 1 h.

The assay specificity for the anti-CpsB antibodies detected was tested in various ways, with the following results: (1) The isotypes of immunoglobulin detected by use of purified CpsB from 3 strains of N. meningitidis or non–O-acetylated CpsK1 of E. coli did not differ significantly; (2) CpsB digestion with neuraminidase before coating abolished antibody detection; (3) competition with anti-
CpsB IgM MAb at concentrations >10 µg/mL totally inhibited the immune IgG binding to CpsB, whereas IgM MAb of unrelated specificity failed to do so; (4) adsorption of serum with N. meningitidis group B or E. coli K1 strains expressing non–O-acetylated CpsK1 removed all anti-CpsB IgG and IgM detectable by ELISA; and (5) the non–O-acetylated CpsK1 in solution competitively inhibited the binding of antibodies to immobilized CpsB at concentrations ranging from 21 to 635 ng/mL (50% inhibition), depending on the immunoglobulin isotype and serum sample tested.

The ELISA described above was transformed into a quantitative assay by introducing appropriate standards in each plate. For anti-CpsB IgM quantitation, 2-fold dilutions of equimolar mixtures of 5 IgM anti-CpsB MAb standards (MGB11, MGB12, MGB13, MGB14, and MGB15), ranging from 1 to 250 ng/mL, were dispensed in CpsB-coated wells and were used as a standard. For all IgG subclass and IgA anti-CpsB quantitations, the only standard used was an equimolar mixture of 2 anti-CpsB IgG2b MAbs (MGB16 and MGB9), ranging from 3 to 500 ng/mL, dispensed in 2-fold dilutions in CpsB-coated wells. The use of a single subclass standard to quantitate heterologous IgG subclasses is possible if one takes the approach of the equivalence dilutions of secondary reagents, which was initially developed to quantitate IgG subclasses in systems lacking calibrated antigen–specific standards for each IgG subclass [22]. The method was based on the precalibration of each secondary anti-isotype reagent, with respect to the anti-κ reagent, to give the same absorbance values and parallelism of the respective titration curves of the primary antibodies.

In this quantitative ELISA, the monoisoantotypic IgG2b standard was incubated with the anti-κ secondary reagent and the samples with the anti-κ subclass-specific secondary reagents, both at their equivalence dilutions. The concentrations of anti-CpsB antibodies of each isotype expressed in weight units were obtained by interpolation of the absorbance values into their respective standard curves [22]. The total IgG anti-CpsB content of the serum was calculated as the sum of the individual concentrations of each IgG subclass. The assay had a detection capacity of 65–48, 95, and 62 ng/mL for the IgG, IgA, and IgM subclasses, respectively.

Levels of anti-CpsC antibodies were obtained by heterologous interpolation of the absorbance obtained in CpsC-coated wells into the curves constructed with the CpsB antibody standards in CpsB-coated wells and are therefore expressed as microgram equivalent per milliliter (µg eq/mL). The levels of antibody specific for OMPs were expressed in arbitrary units, with 1 anti-OMP unit, considered to be the content of a particular isotype in the undiluted polyclonal ascitic fluid against N. meningitidis group B, used as the standard in the OMP-specific ELISA. This was assayed in a manner similar to the Cps ELISA, except that the plates were coated directly with purified meningococcal OMPs diluted to 10 µg/mL in PBS.

The results are presented as the geometric mean ± SD of the individual concentrations. Levels were compared using Student’s 2-tailed t test. P < .05 was considered to be significant.

Results

Ontogeny of the natural antibodies specific for meningococcal Cps.

In our initial studies, we determined the titers of antibody reactive with either CpsB or CpsC in preimmune serum samples of mice at 0–14 weeks of age (figure 1). We used these data to establish a baseline for assessing induction of anti-CpsB or anti-CpsC antibodies in response to intact N. meningitidis. In preimmune serum samples, CpsB-specific antibodies were barely detectable in the serum of 12-day-old mice, but they increased subsequently to a maximum concentration of 0.5 ± 0.15 µg/mL by 5–6 weeks of age. This was followed by a progressive decrease to basal levels at 11 weeks (0.27 ± 0.09 µg/mL), which persisted throughout adulthood. Only the IgMc anti-CpsB isotype was detectable. No IgG or IgA was detected.

The development of natural anti-CpsC antibodies showed 2 major differences from that observed for the anti-CpsB antibodies (figure 1). First, IgG2a anti-CpsC, in addition to IgM, was detectable in the preimmune serum samples from 6- to 7-week-old mice. These IgG2a levels increased to 0.25 ± 0.1 µg/mL at 9–10 weeks and were detectable in 80% of the mice in this age group. Furthermore, the level of IgG2a anti-CpsC decreased with age to the adult level of 73 ± 28 ng eq/mL. No other CpsC-specific IgG subclass was detectable throughout the age ranges tested. Second, natural anti-CpsC IgM levels underwent little variation with age, except for an isolated peak of IgM anti-CpsC levels at 7–8 weeks.

E. coli K1 or other endogenous aerobic bacteria expressing antigens cross-reactive with CpsB were not components of the mouse microflora. To test the hypothesis that cross-reactive antigens expressed in host-colonizing organisms induce natural anti-CpsB antibodies, we attempted to isolate them from the normal murine flora. Bearing in mind the age-dependent profile

![Figure 1](image_url). Ontogeny of natural antibodies specific for the capsular polysaccharides B (CpsB; left) and C (CpsC; right) in BALB/c mice. White bars indicate IgM levels and black bars indicate IgG2a levels present in the preimmune serum of 10 mice for each age group (geometric mean ± SD).
of the natural antibody levels and the fact that their initial appearance in serum at 2–3 weeks of age coincides with the onset of solid food consumption, a broad age range (2–10 weeks) of mice was sampled to consider the possible influence of alterations in their intestinal microflora on natural anti-CpsB responses: 20 mice were sampled from each age group. *E. coli* was almost always isolated from cultures of intestinal and fecal origin. This was evident from colony morphology (pink, nonmucoid) on MacConkey selective media and by eventual isolation of colonies reactive with an antiserum specific for *E. coli* antigen O8 in colony-blot assays. However, we could not identify *E. coli* isolates expressing CpsK1 or bacteria reactive with anti-CpsB antibodies from any sample source. Even if several colonies isolated from nasopharyngeal swab samples were positive for the polyclonal ascitic fluid against *N. meningitidis* group B, this reactivity was due to subcapsular antigens, since they did not react with anti-CpsB MAb on primary isolation or after subculture. Our mouse colony seemed, therefore, to be free of aerobic bacteria containing CpsB cross-reactive antigens, and *E. coli* K1 is not a component of their flora.

**Ontogeny of the anti-CpsB IgM response to bacterial immunization.** The ability to produce an IgM anti-CpsB antibody response after immunization with whole bacteria started early in ontogeny (figure 2) and showed a pattern similar to that observed for natural anti-CpsB antibodies (figure 1). In 3-week-old mice, the IgM response elicited by a single immunization with $2 \times 10^8$ cfu of CpsB was already 10-fold higher than the natural antibody levels in the serum of unimmunized mice. The elicited anti-CpsB IgM response increased progressively with age up to 9 weeks, at which time it was fully developed. At this age in mice, the anti-CpsB IgM response reached 50 ± 7 μg/mL. No further changes were seen in older mice. Conversely, none of the age groups developed specific antibodies after intraperitoneal or intrasplenic immunization with purified CpsB (1 or 10 μg; data not shown).

Previous stimulation with antigen in the form of whole bacteria affected the response obtained after secondary immunization in a different way, depending on the age at which the animal was immunized and the bacterial dose used. Mice in each age group immunized 2 weeks earlier with the same dose ($2 \times 10^8$ cfu of CpsB) produced IgM anti-CpsB levels in the secondary response similar to those of mice receiving their primary immunization at the same age. This was true for all except the earlier age group (3 weeks), in which a significant decrease in IgM anti-CpsB levels was observed in mice previously immunized at 1 week of age. Therefore, no anamnestic IgM response was obtained. This strongly suggests that the age-related increase in primary IgM anti-CpsB responses to bacteria is not due to anamnestic responses in naturally primed mice. The use of a higher bacterial dose ($10^{10}$ cfu) for secondary immunization elicited 3–4-fold higher IgM anti-CpsB antibody levels than those elicited by the low bacterial dose in mice about or >7 weeks of age ($P < .0002$; figure 2). However, in mice <5 weeks old, secondary immunization led to lower IgM

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**Figure 2.** Ontogeny of the antibody responses for the capsular polysaccharide B (CpsB) induced by bacterial immunization. Data are IgM (left) and IgG (right) CpsB-specific serum antibody levels 5 and 6 days, respectively, after primary immunization with $2 \times 10^8$ cfu of CpsB (white bars) and 5 and 6 days, respectively, after challenge with the same dose (hatched bars) or a dose of $10^{10}$ cfu (black bars) 2 weeks later. Antibody concentrations are geometric mean ± SD for 20 serum samples from each age group except 16-week-old mice. The corresponding preimmune levels of anti-CpsB antibodies shown in figure 1 were subtracted.
responses than those observed during the primary immunization (\(P < .006\)). Thus, secondary immunization with high bacterial doses may exert both positive and negative effects on the IgM response, depending on whether the mice are mature or immature for anti-CpsB responses.

Immunization with \(N.\) meningitidis group B did not noticeably affect natural anti-CpsC levels, except for an initial delay in the appearance of natural anti-CpsC antibodies in mice immunized during the perinatal period (figure 3). Similarly, immunization with group C meningococci did not modify the levels of natural anti-CpsB antibodies. Therefore, anti-CpsB IgM elicited by bacterial immunization did not cross-react with CpsC.

**Ontogeny of the anti-CpsB IgG response to bacterial immunization.** The IgG anti-CpsB immune response, compared with the IgM response, was delayed in ontogeny (figure 2). The IgG anti-CpsB response first became detectable at 7 weeks of age in 20% of mice immunized with \(2 \times 10^8\) cfu of CpsB. The IgG levels elicited by the primary immunization and the percentage of IgG responders increased with age, reaching 80% at 14 weeks of age (figures 2 and 4). Unlike IgM responses, previous stimulation with the same bacterial dose (\(2 \times 10^8\) cfu of CpsB) produced significant increases in IgG anti-CpsB levels elicited after secondary stimulation, except at the terminal period of the response development (7 weeks). At this age in mice, the IgG levels did not differ significantly (\(P = .77\)) from those obtained in mice of the same age after primary immunization (figure 2). The secondary IgG response was also dose dependent. Nevertheless, when the immunizing dose was \(10^{10}\) cfu of CpsB, the increase was \(\sim 10–15\)-fold higher than that obtained after the primary immunization of age-matched mice with \(2 \times 10^8\) cfu. Moreover, immunization with this bacterial dose during the response maturation period induced earlier IgG responses.

Mice challenged with \(10^{10}\) cfu at 5 weeks of age produced anti-CpsB IgG, and significant increases in the IgG levels were observed at 7 weeks of age. It was remarkable that IgG/IgM anti-CpsB ratios during this period were considerably higher than those in mice with developmentally mature anti-CpsB responses. The mean ratio of anti-CpsB IgG/IgM was 0.42 at 5 weeks of age, 0.11 at 7 weeks, and only 0.038 at 16 weeks. IgA anti-CpsB responses were negligible at all ages.
Ontogeny of the IgG subclass anti-CpsB response. The IgG subclass distribution undergoes progressive change during the development of the anti-CpsB responses (figure 4). The IgG subclass response of 5-week-old mice was predominantly IgG3, with some IgG1. The IgG subclass profile, progressively changed with the mice’s age, and responses in 9-week-old mice were dominated by IgG2a and IgG2b (figure 4B). It is interesting that the reverse IgG change (i.e., mostly IgG2a/IgG2b to IgG3/IgG1) was induced in 9-week-old mice by hyperimmunization [20].

Mouse sex does not influence the development of the anti-CpsB response. IgG and IgM anti-CpsB antibody responses did not show significant differences between the sexes at any age tested (3–11 weeks; P > .05). Thus, differences in sexual maturation do not influence the ontogeny of the anti-CpsB antibody response.

Influence of hyperimmunization on development of the anti-CpsB antibody response. As we reported earlier [20], hyperimmunization with whole bacteria of mice >9 weeks of age induced high CpsB-specific antibody responses, changed the IgG subclass distribution toward IgG1/IgG3, and generated B-cell memory to further suboptimal challenge [20]. In an attempt to elucidate whether continued stimulation with a competent immunogenic form of the CpsB might have similar effects on mice during ontogeny of the anti-CpsB response, we hyperimmunized mice at 4, 6, and 7 weeks of age (figure 5). However, 60% (8/13) of mice at 4 weeks of age did not produce detectable anti-CpsB antibodies during the entire hyperimmunization regimen, even though the last challenge was administered at 8 weeks of age. An IgM anti-CpsB response was observed in the remaining mice (5/13); however, 4 of the 5 did not produce detectable antibody responses after immunizations at 5–6 weeks of age. At the end of the schedule, IgM levels were lower than those elicited by a single immunization at 8 weeks of age (2.3 ± 0.4 vs. 32 ± 9 μg/mL). None of the mice produced anti-CpsB IgG.

The 6-week-old group showed intermediate behavior. Only 1 mouse did not produce an antibody response throughout the schedule (other than a low IgM response after initial immunization). However, the most frequent pattern (in 60% of mice) was a decrease in antibody levels as the immunization schedule progressed (figure 5), a decrease that was suppressed in 25% of the mice after the second week of immunization (at 8 weeks of age). In 30% of the remaining mice, the anti-CpsB antibody levels underwent a constant increase during hyperimmunization but were lower than those in mice receiving a single immunization at the same age (figure 2). This last profile was observed in 80% of the 7-week age group (figure 5). The remaining 20% underwent the attenuation of the anti-CpsB antibody levels observed in the preceding age group.

An IgG response was detected in 44% and 60% of mice in the 6- and 7-week age groups, respectively. Although these IgG levels 5–7 days after the last immunization were lower than those in mice hyperimmunized at 9 weeks of age (7.6 ± 4.2 vs. 11.6 ± 6.6 μg/mL), their IgG/IgM ratio was 0.87, 8-fold higher than that for mice hyperimmunized at 9 weeks of age, with an IgG/IgM ratio of 0.11. In addition, IgG became dominant in subsequent blood samples (figure 5). Continuous stimulation, therefore, affects anti-CpsB responses differently, depending on the developmental stage. In the early stages, it induces unresponsiveness, and in the later stages, it both attenuates the IgM responses and favors a switch from IgM to IgG.

The serum anti-OMP and anti-CpsC antibody profiles clearly differed from those described above for the anti-CpsB response (figure 5). Although an ontogenic delay was observed in the anti-CpsC response of the 4-week age group, unresponsiveness or attenuation was not observed at any age. Moreover, the elicited anti-CpsC antibodies were IgG in all groups, and their serum profiles resembled those observed in the T cell–dependent, anti-OMP responses. The serum anti-OMP responses were unexpectedly higher in younger mice.

Discussion

After immunization with whole bacteria, CpsB-specific antibody responses peak for 5 days, and the elicited antibodies are rapidly cleared from the bloodstream [20]. These dynamics allow for more accurate interpretation of the study of the development of the CpsB antibody responses during ontogeny, because they minimize the effects derived from the maturation of the immune system during the elicitation of the response.

The ability to produce an IgM anti-CpsB response in mice starts at ~3 weeks of age and reaches complete development ~9 weeks after birth. In humans, IgM anti-CpsB antibodies can be induced in the serum of 6–7-month-old patients infected with N. meningitidis B [7]. The age at which the human anti-CpsB antibody response reaches maturity has not been clearly defined, although this may occur around puberty [7, 25]. Thus, the ontogeny of the anti-CpsB antibody response in mice shares similarities with that in humans.

Two major types of T cell–independent (TI) antigens have been defined on the basis of their ability (type 1) or inability (type 2) to induce specific antibody responses in neonates and xid mice [26]. TI type 1 antigens, such as lipopolysaccharides, are mitogens. In contrast, TI type 2 antigens, such as purified polysaccharides, are nonmitogenic polymers with highly repetitive epitopes in their structure. As we observed, despite the immunogenicity of the CpsB presented on the bacterial surface, the purified CpsB did not induce antibody responses at any age. This strongly suggests that adjuvant factors, such as bacterial lipopolysaccharides, OMPs, and lipoproteins, play an important role in costimulating the secretion of anti-CpsB antibodies by CpsB-activated B cells. Even so, these stimuli did not significantly induce antibody responses earlier in ontogeny, relative to TI type 1 or T cell–dependent responses.
Thus, the presence of mitogens in bacteria does not invariably lead to TI type 1 behavior of multivalent antigens. In this sense (as we described earlier), the response to CpsB but not to CpsC on the bacterial surface is TI type 2 [20]. Moreover, the ontogenic pattern of the CpsB response was similar to that of responses to most purified Cps. A similar pattern, except for the earlier mature responses (at 6–7 weeks), has been observed to purified CpsA and CpsC [2, 27]. Hence, the delayed onset of the anti-CpsB responses in ontogeny seems to bear more relationship to mechanisms common to responses to TI antigens.

Particular aspects of the CpsB immunobiology may determine the delay in attaining mature responses, even in the presence of bacterial adjuvant molecules, as well as the unresponsiveness to purified CpsB. It could be hypothesized to be due to a deficient B-cell maturation or clonal abortion of immature CpsB-specific B cells by the contact with endogenous polysialic acid during the perinatal period. In newborn mice, polysialic
Natural anti-CpsB antibodies are considered to be induced during the asymptomatic carriage of *N. meningitidis* group B or of microorganisms expressing cross-reactive Cps. Nevertheless, only humans carry *N. meningitidis*, and, in general, their carriage does not increase serum anti-CpsB antibody levels [10]. Among bacterial species described that express Cps identical to the CpsB, all of which are aerobes or facultative aerobes, only *E. coli* K1 has sufficient dissemination in vertebrates to justify the frequency of natural anti-CpsB antibodies and explain its presence in every vertebrate species tested [11, 12]. However, we could not demonstrate *E. coli* K1 or other nonanaerobic bacteria expressing cross-reactive antigens in the microflora of our mouse colony, despite the broad range of ages and mucosal epithelia tested. In adult mice, ~95% of the gastrointestinal flora are obligate anaerobes [2]. Moreover, negative results are frequent. Rubinstein and Stein [2] could not isolate bacteria expressing Cps cross-reactive with CpsC. However, it is unquestionable that genetic background and environmental stimuli influence natural antibodies specific for saccharide antigens. Thus, the natural anti-CpsC IgG was restricted here to IgG2a, as found by Moreno and Esdaile [33], but differs from the IgG3 restriction described by Rubinstein and Stein [2].

It is unlikely, however, that exogenous antigens can be the major stimulus for anti-CpsB natural antibody induction. The developmental appearance of natural anti-CpsB antibodies even precedes the development of anti-CpsB antibody responses to bacterial immunization, reaching a maximum during terminal maturation of the response. Competent stimulation with CpsB during this period induced unresponsiveness rather than specific responses. Polyclonal activation of the reactive B cells by subcapsular antigens is also unlikely. Thus, natural anti-CpsB antibody levels were not significantly modified by immunization with *N. meningitidis* C.

The profile of the appearance of natural anti-CpsB antibodies in ontogeny could be interpreted to be the result of the expression during ontogeny of representative sequences of the variable region germline genes coding for autoreactive specificities, as has been proposed for natural autoantibodies [34]. Recombination and somatic mutation will generate additional specificities, with the consequent germline sequence dilution. The high frequency of paraproteins with CpsB specificity idiotypically related to autoantibody specificities and the high homology of their variable regions with the respective germline genes support the model [35]. The plausible absence of exogenous stimuli in our mouse colony could favor the observed reduction of natural anti-Cpb antibody levels with age. Thus, natural anti-CpsB antibodies could be considered to be autoantibodies, components of the autoreactive idiotypic network active in unimmunized animals that contribute to homeostasis [36].

In summary, our data demonstrate levels of complexity in the ontogeny of the humoral response to *N. meningitidis* B that...
should be considered in the evaluation of the efficacy of vaccine candidates. These factors include (1) the increase in the anti-CpsB antibody response with age; (2) the delay in attaining mature responses; (3) the positive or negative effects that the dose and persistence of the stimulation have over the anti-CpsB response, depending on the maturation level attained; (4) the changes in the IgG subclass distribution; and (5) the higher relative proportion of specific IgGs produced during the terminal period of development. This is particularly so when the reinforcement, IgG/IgM ratios, or IgG subclass distributions of the secondary antibody responses are used as major indicators of the efficacy and thymus dependence of the response to the vaccine. On the other hand, vaccination at earlier ages could reduce the anti-CpsB response during a later natural infection with *N. meningitidis*. This also could be important when serial immunizations occur. On the contrary, the anti-CpsB antibody response properties during terminal development (5–7 weeks of age in mice, which could correspond to 2–5 years of age in humans) could favor the isotype switch and therefore become advantageous. However, its utility resides in 2 aspects that are still obscure, namely, the safety of the anti-CpsB antibodies, particularly IgGs, and the greater efficacy of IgG antipolysaccharide responses.

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**References**


