Journal of Clinical Laboratory Analysis 21:201–206 (2007)

Improved Method to Calculate the Antibody Avidity Index

C.T. Perciani,[1] P.S. Peixoto,[2] W.O. Dias,[1] F.S. Kubrusly,[1] and M.M. Tanizaki[1]*
[1]Centro de Biotecnologia, Instituto Butantan, São Paulo, Brazil
[2]Instituto de Matemática e Estatística, University of São Paulo, São Paulo, Brazil

The methods currently used to determine the immunoglobulin avidity index (AI) require the choice of a reference point in the ELISA titration curve. Since both curves, with and without denaturating reagents, seldom run in parallel, the AI value becomes highly dependent on this reference. The new method for AI calculation presented here takes into account the whole data of the ELISA titration curve in which the final numerical AI is the average of each point. J. Clin. Lab. Anal. 21:201–206, 2007. © 2007 Wiley-Liss, Inc.

Key words: oil/water emulsion; porcine pulmonary surfactant; outer membrane vesicle; meningococcal vaccine

INTRODUCTION

During the maturation of the humoral immune response, there is an antibody selection process that results in synthesis of antibodies with increased antigen-antibody association strength. The numerical constant that represents this association strength is the avidity index (AI). Analysis of IgG avidity proved to be useful for differentiating primary and secondary infections in many infectious diseases, such as toxoplasma, rubella, and mumps (1–3). In addition, the determination of antibody avidity has been gaining importance in the assessment of vaccine efficacy, where the induction of high-avidity antibodies is desired (3,4). The AI is currently measured in immune sera by enzyme-linked immunosorbent assay (ELISA) with the inclusion of one additional step: treatment of the antigen-antibody complex with denaturating reagents, such as urea or thiocyanate (1,2). Experimentally, AI is usually determined by varying the antibody concentration; alternatively, it is also possible to vary the concentration of denaturating reagent (5). The latter method is used less frequently, probably because it consumes more time.

Although the assay used to determine antibody avidity is a very well established procedure, the methods for AI calculation are still controversial. AI calculation has been performed in numerous ways, all of which are based on the ratio or percentage of bound antigen in the ELISA plate with and without a denaturating agent, which would be expressed as the optical density (OD) (4,6), titer (7), or graphical distance (shift) between both titration curves (8). When the AI is expressed as the ratio of OD, usually a single-point determination in which one fixed standard OD is used. Otherwise, when the AI is expressed by the ratio of antibody titer, the whole ELISA titration curve should be performed. In this study we tested two methods for AI calculation: OD and titer. We used sera of newborn mice vaccinated with an anti-meningococcal serogroup B vaccine with an oil/water emulsion prepared with porcine pulmonary surfactant (PPS) (9) as adjuvant.

Vaccines against *Neisseria meningitidis* serogroup B using outer membrane vesicles (OMV) as antigen have low efficacy in infants (10,11) because they induce the production of antibodies with low avidity, which do not present bactericidal activity (12,13). In a previous study (15) we showed that OMV-capsular polysaccharide conjugated vaccine added to MF59®, an oil/water type adjuvant (14), was able to induce antibodies against OMV with increased avidity and bactericidal activity in newborn mice. In the present study we tested an alternative adjuvant hoping to induce similar high-avidity antibodies in newborn mice. This adjuvant was

*Correspondence to: M.M. Tanizaki, Centro de Biotecnologia, Instituto Butantan, Avenida Vital Brasil 1500, São Paulo, Brasil, CEP 05503-900. E-mail: tanizaki@butantan.gov.br

Grant sponsor: Fundação de Amparo a Pesquisado Estado de São Paulo (FAPESP); Grant sponsor: Conselho Nacional de Pesquisa e Desenvolvimento.

Received 17 November 2006; Accepted 9 March 2007
DOI 10.1002/jcla.20172
Published online in Wiley InterScience (www.interscience.wiley.com).

also an oil/water emulsion prepared with PPS. Using the data of this experiment, we noticed imprecision in the AI calculation using the two methods. The imprecision was due the fact that calculation of AI by these methods involves the choice of a reference point in the ELISA titration curve. However, titration curves obtained by ELISA with and without denaturating reagents seldom run in parallel (6,7). As a consequence of this unparallel profile, AI values become highly dependent on the reference point used for their calculation. Therefore, we deduced a new method for AI calculation aiming for more precision. This new method is based on the ratio of the area value obtained in the ELISA graphic (OD vs. log of dilution) with and without treatment with a denaturating reagent. With this method, it was possible to obtain more accurate results in the AI calculation.

MATERIALS AND METHODS

Mice

Newborn Swiss mice (5 days old, about seven animals per group) were maintained in the animal facility during the experiments. For the generation of newborn mice, pregnant females were separated and caged individually, and newborns were maintained with their mother during the experiment.

Vaccine, Adjuvant, and Immunization Scheme

OMV was produced according to a previously described method (15). The oil/water PPS was prepared using 5% squalene, 0.5% Tween 80, and 0.5% of PPS in PBS. The solution was homogenized by vortex in five cycles of 2 min, followed by 10 cycles of 1 min of sonication (Branson Sonifier 450) with intervals of 3 min between each cycle in an ice bath. The vaccines were formulated using 0.2 μg of OMV plus 20 μg of aluminum hydroxide or PPS emulsion in a proportion of 1:1 (v:v). The group of animals was injected with 1) OMV plus aluminum hydroxide, 2) OMV plus PPS emulsion, and 3) saline, by the intraperitoneal route, using 0.05 mL per dose. A booster dose was administered 1 week afterwards and the mice were bled by retro-orbital puncture at 4 weeks after the first injection.

ELISA and AI Determination

Antibody avidity was measured using thiocyanate (KSCN) as the chaotropic agent (16). In short, Hybond Nunc 96 plates were coated overnight with 100 μL of a 4 μg/mL OMV solution in 100 mM Tris/HCl buffer, pH 8.5. Sera were diluted in PBS in quadruplicate and incubated for 90 min at 37°C. After three washes with PBS, 100 μL of KSCN 1.5 M dissolved in PBS were added to one-half of the serial dilution and plain PBS was added to the other one-half plate. After a 15-min incubation, all wells were washed three times with PBS, incubated with anti-mouse IgG-horseradish peroxidase and revealed with 3,3′,5,5′-tetramethylbenzidine substrate.

AI was determined by three different methods: 1) single-point determination (4), in which the OD492nn obtained by thiocyanate treatment was divided by the respective OD492nm of the control assay, which was settled as 1.0 and 0.5; 2) endpoint titer determination, in which the endpoint titer was determined as the sera dilution OD492nm = 0.5 and 0.2; and 3) the area of the antibody titration curve, as described in this article.

Calculation of AI Based on the Area of Antibody Titration

The method proposed here is based on the ratio of the areas derived from the curves obtained by the plot of OD and log of the sera dilution in the ELISA experiment with and without thiocyanate treatment.

Figure 1 shows a typical plot of ELISA with and without a KSCN wash, and the AI was defined by the following equation:

$$AI = \frac{A_K}{A_C} \tag{1}$$

where A_K is the area under the KSCN-washed curve, and A_C is the area under the control curve. In this method the area calculation was divided into a sum of trapezoid areas (Fig. 1), and each trapezoid's area was calculated as follows:

$$\begin{aligned} A_{xi} &= h(a_{x,i+1}) + h\left(\frac{a_{x,i} - a_{x,i+1}}{2}\right) \\ &= \frac{h}{2}(a_{x,i} + a_{x,i+1}) \end{aligned} \tag{2}$$

In which x assumes c or k, for control or KSCN curves, respectively; A_{xi} is the area of the ith trapezoid relative to either control (A_{ci}) or KSCN (A_{ki}) curves; $i = 1, 2, \ldots, n–1$; n is the number of dilutions; h is the distance between the parallels of the trapezoid, and shall be constant for all trapezoids in experiments with logarithmic dilutions; and $a_{x,i}$ is the OD related to the i^{th} sample, or for the i^{th} dilution, and the x type of curve.

Adding all trapezoids, we obtain

$$A_x = \sum_{i=1}^{n-1} A_{xi} = \frac{h}{2}\sum_{i=1}^{n-1}(a_{x,i} + a_{x,i+1}) \tag{3}$$

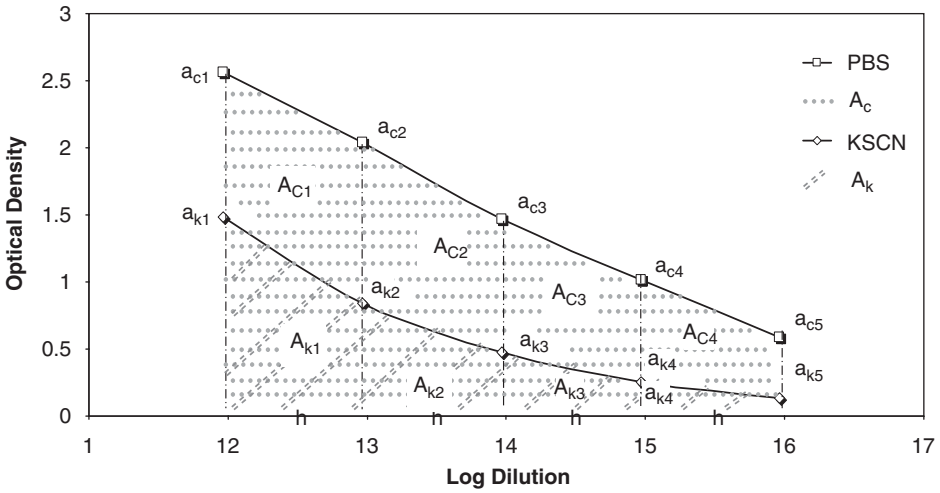

Fig. 1. Typical plot of ELISA with and without a KSCN wash used to calculation of AI based on the Area of Antibody Titration. Squares, OD of PBS curve (a_{Ci}); diamonds, OD of KSCN curve (a_{Ki}); A_{Ci}, area under KSCN curve.

That leads us to the final formula of the AI (Eq. [1]), using Eq. [3], with simplifications:

$$AI = \frac{A_k}{A_c} = \frac{\frac{h}{2}\sum_{i=1}^{n-1}(a_{k,i} + a_{k,i+1})}{\frac{h}{2}\sum_{i=1}^{n-1}(a_{c,i} + a_{c,i+1})} = \frac{2\sum_{i=1}^{n} a_{k,i} - a_{k,1} - a_{k,n}}{2\sum_{i=1}^{n} a_{c,i} - a_{c,1} - a_{c,n}} \quad (4)$$

According to Eq. [4], the AI can be easily calculated as the ratio of two times the summation of the ODs obtained in the ELISA plot under the denaturating condition minus the ODs of its first and last titration data, divided by two times the summation of the ODs obtained in the ELISA plot under the control condition (without denaturation) minus the ODs of its first and last titration data. This is a simplified numerical way to calculate the relationship between areas of curves instead of using integral functions.

RESULTS

The oil/water surfactant used in these experiments was formulated based on the adjuvant MF59®, in which only one component (Span 80) was substituted by PPS. PPS is a mixture of phospholipids in which the main component (about 76%) is phosphatidylcholine (10). It matched very well with squalene and Tween 80 to form a stable emulsion. The PPS emulsion was tested as adjuvant with an anti-meningococcal OMV vaccine using the same protocol previously tested with MF59® (15). Neonate mice were injected with OMV antigen plus PPS emulsion or aluminum hydroxide as control. At the end of the immunization schedule, the mice were bled and the sera analyzed for IgG avidity.

In Fig. 2 the OD 492nm values of ELISA from each mouse were plotted against the respective log of dilutions, and these plots were used to calculate the AI. As expected, in most of the plots the thiocyanate-treated and control curves were not parallel. As a consequence of this unparallel shape, the AI values were highly dependent on the reference value chosen for their calculation, and Table 1 shows the magnitude of this variation. In order to demonstrate the variability of AI, the values were determined in individual mice sera by the three methods described in the Materials and Methods section. According to Table 1, AI calculation was over- or underestimated depending on the choice of the standard reference. Furthermore, at a low reference value, such as the sera dilution that resulted in an OD492 = 0.2 (Table 1, method 2), samples with low IgG avidity could not be determined or were imprecisely determined.

DISCUSSION

Different trials have shown that meningococcal vaccines constituted by OMV are efficient for adults and children older than 2 years of age, but not for infants (16,18). One reason for the failure of the vaccine in young children seems to be the difference in some cytokine production profiles in young children (20). Furthermore, there is also a difference in the quality of the antibodies in young children, since the serum bactericidal activity of IgG is also low (20). The effective anti-meningococcal antibodies are related to high avidity and complement dependent bactericidal activity (21). We previously showed that the oil/water adjuvant

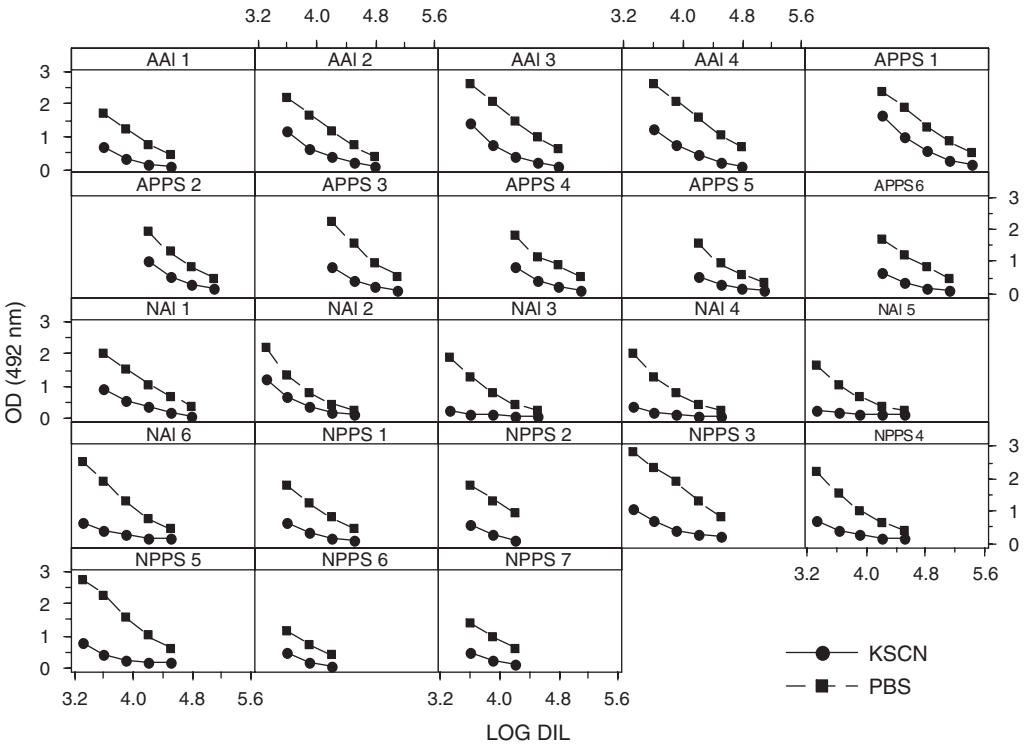

Fig. 2. IgG ELISA profile of control and KSCN treated sera of mice immunized with OMV vaccine. AAl, sera of adult mice immunized with OMV and Aluminum hydroxide; APPS, sera of adult mice immunized with OMV plus pulmonary surfactant emulsion; NAl, sera of newborn mice immunized with OMV plus aluminum hydroxide; NPPS, sera of newborn mice immunized with OMV plus porcine pulmonary surfactant.

TABLE 1. AI determination using three different methods*

Mice	Method 1 (OD = 1)[a]	Method 1 (OD = 0.5)[a]	Method 2 (OD = 0.5)[b]	Method 2 (OD = 0.2)[b]	Method 3 (areas)
AAl 1	0.29	0.07	0.22	0.36	0.27
AAl 2	0.34	0.11	0.31	0.44	0.37
AAl 3	0.22	n.d.	0.28	n.d.	0.34
AAl 4	0.18	n.d.	0.23	n.d.	0.31
APPS 1	0.42	0.07	0.43	n.d.	0.49
APPS 2	0.41	0.23	0.40	0.54	0.41
APPS 3	0.25	0.06	0.27	0.44	0.28
APPS 4	0.34	0.11	0.28	n.d.	0.34
APPS 5	0.30	0.23	0.17	0.40	0.28
APPS 6	0.28	0.14	0.19	0.37	0.27
NAl 1	0.34	0.16	0.26	n.d.	0.35
NAl 2	0.51	0.43	0.49	0.67	0.50
NAl 3	0.14	0.16	0.00	0.11	0.14
NAl 4	0.17	0.18	0.01	0.20	0.16
NAl 5	0.18	0.25	0.00	n.d.	0.20
NAl 6	0.20	0.16	0.11	0.14	0.20
NPPS 1	0.24	0.08	0.17	0.35	0.24
NPPS 2	0.11	n.d.	0.15	0.22	0.21
NPPS 3	0.16	n.d.	0.17	n.d.	0.26
NPPS 4	0.27	0.23	0.16	n.d.	0.26
NPPS 5	0.19	0.10	0.13	n.d.	0.21
NPPS 6	0.37	0.17	0.25	0.39	0.30
NPPS 7	0.27	0.04	0.17	0.30	0.27

*AI was determined using three different methods: Method 1, single-point determination; Method 2, end-point titration using; Method 3, based on titration area.
[a]Cutoff OD.
[b]Titer calculation corresponded to OD.
Aal, sera of adult mice immunized with OMV vaccine and aluminum hydroxide as adjuvant; APPS, sera of adult mice immunized with OMV vaccine and porcine surfactant emulsion as adjuvant; NAI, sera of newborn mice immunized with OMV vaccine and aluminum hydroxide as adjuvant; NPPS, sera of newborn mice immunized with OMV vaccine and porcine surfactant emulsion as adjuvant; n.d., not determined.

MF59 included in the OMV vaccines was able to induce high-avidity antibodies with bactericidal activity in newborn mice (15). MF59 is an oil/water emulsion composed of squalene, Tween 80, and Span 85 in water (14). In this study we tested an emulsion in which Span 85 was replaced by PPS. In its formulation, the amount of squalene, which is considered the main component of MF59, was maintained and a stable emulsion was obtained. However, as shown in this study, the PPS emulsion when added to the OMV vaccine failed to induce high-avidity antibodies in newborn mice. Working with the sera of mice immunized with OMV vaccine, whose results are presented here, we noticed that the main cause of the variation in AI determination is that all of the methods require the choice of a reference point in the ELISA titration curve. The shape of the ELISA graphical plot of KSCN-treated and -untreated samples seldom runs as parallel curves (7,8), probably due to the fact that these reagents inhibit the antigen–antibody interaction in a competitive mechanism. Therefore, the AI value will be over- or underestimated depending on the reference point used for its calculation. This unparallel shape is more emphasized in samples with low AI (7), and as consequence the imprecision in AI evaluation is higher in serum samples with low avidity than with high-avidity antibodies. The reference points used here and shown in Table 1 are the same as those used in the literature: I) ODs corresponding to 0.2 or 0.1 are currently the choice for the single-point determination method (7); II) a serum dilution corresponding to OD = 0.1 or 0.2 was also taken in the endpoint titration method (7,22); III) and a value that was 50% of the maximum OD, which corresponds to about 0.8 to 1.0, was also used (23,24).

The AI calculated by the method of area is reliable because it takes into account the data of the whole ELISA titration curve, where the final numerical AI is the average of each point. In order to make the area calculation a simple process, the hyperbolic shape was substituted by a sequence of trapezoids. Therefore, instead of an integral calculation, the final numeric AI is the ratio (KSCN-treated vs. control) of the summation of the trapezoids obtained from the titration curve. A reliable method to calculate the antibody AI is highly desirable for laboratory clinical diagnosis to discriminate recent from distant infection in many diseases, and the method presented here may result in less imprecision than the current ones.

ACKNOWLEDGMENTS

C.T. Perciani is the recipient of a fellowship from the Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP).

REFERENCES

1. Hedman K, Lappalainen M, Seppaia I, Makela O. Recent primary toxoplasma infection indicated by a low avidity of specific IgG. J Infect Dis 1989;159:736–740.
2. Wilson KM, Di Camili C, Doughty L, Dax EM. Humoral response to primary rubella virus infection. Clin Vaccine Immunol 2006;13:380–386.
3. Goldblatt D, Vaz AR, Miller E. Antibody avidity as a surrogate marker of a successful priming by *Haemophilus influenzae* type b conjugate vaccines following infant immunization. J Infect Dis 1998;177:1112–1115.
4. Vermont CL, van Dijken HH, van Limpt CJ, de Groot R, van Alphen L, van Den Dobbelsteen GP. Antibody avidity and immunoglobulin G isotype distribution following immunization with a monovalent meningococcal B outer membrane vesicle vaccine. Infect Immun 2002;70:584–590.
5. Gioia CAC, Sousa AB, Cruz SC, et al. Effect of booster dose of serogroup B meningococcal vaccine no antibody response to *Neisseria meningitidis* in mice vaccinated with different immunization schedules. FEMS Immunol Med Microbiol 2005;44: 35–42.
6. Gassman C, Bauer G. Avidity determination of IgG directed against tick-borne encephalitis virus improves detection of current infections. J Med Virol 1997;51:242–251.
7. Jenun PA, Stray-Pedersen B, Gundersen AG. Improved diagnosis of primary *Toxoplasma gondii* infection in early pregnancy by determination of antitoxoplasma immunoglobulin G avidity. J Clin Microbiol 1997;35:1972–1977.
8. Alvarado-Esquivel C, Sethi S, Janitschke K, Hahn H, Liesenfeld O. Comparison of two commercially available avidity tests for *Toxoplasma*-specific OgG antibodies. Arch Med Res 2002; 33:520–523.
9. Korhonen MH, Brunstein J, Haario H, Katnikiv A, Rescaldani R, Hedman K. A new method with general diagnostic utility for the calculation of immunoglobulin avidity. Clin Diagn Lab Immunol 1999;6:725–728.
10. Pollard AJ, Levin M. Vaccines for prevention of meningococcal disease. Pediatr Infect Dis J 2000;19:333–345.
11. Milagres LG, Ramos SR, Sacchi CT. Immune response of Brazilian children to a *Neisseria meningitidis* serogroup B outer membrane protein vaccine: comparison with efficacy. Infect Immun 1994;62:4419–4424.
12. Pollard AJ, Galassini R, van der Voort E. Humoral immune responses to *Neisseria meningitidis* in children. Infect Immun 1999;67:2441–2451.
13. Pollard AJ, Levin M. Production of low-avidity antibody by infants after infection with serogroup B meningococci. Lancet 2000;356:2065–2066.
14. Singh M, Ugozzoli M, Kazzaz J, et al. A preliminary evaluation of alternative adjuvants to alum using a range of established and new generation vaccine adjuvants. Vaccine 2005;24:1980–1986.
15. Fukasawa LO, Dias WO, Schenkman PF, Raw I, Tanizaki MM. Adjuvant can improve protection induced by OMV vaccine against *Neisseria meningitidis* serogroups B/C in neonatal mice. FEMS Immunol Med Microbiol 2004;41:205–210.
16. Kubrusly FS, Netto SL, Iortov D, Raw I, Araujo PS. A natural surfactant from pig lungs. Biotechnol Lett 2000;22:1251–1253.
17. Noronha CP, Struchiner CJ, Halloran ME. Assessment of the direct effectiveness of BC meningococcal vaccine in Rio de Janeiro, Brazil: a case control study. Int J Epidemiol 1995;24: 4419–4424.
18. Boslego J, Garcia J, Cruz C. Efficacy, safety and immunogenicity of a meningococcal group B (15:P1.3) outer membrane vesicle vaccine in Iquiuqe, Chile. Vaccine 1995;13:821–829.

19. Tapero JW, Lagos R, Ballesteros AM, et al. Immunogenicity of 2 serogroup B outer membrane protein meningococcal vaccines: a randomized controlled trial in Chile. JAMA 1999;281: 1541–1543.
20. Pollard AJ, Galassini R, Roupe EM, et al. Cellular immune responses to *Neisseria meningitidis* in children. Infect Immun 1999;67:2452–2463.
21. Pollard AJ, Galassini R, Rouppe EM, et al. Humoral immune responses to *Neisseria meningitidis* in chidren. Infect Immun 1999;67:2441–2451.
22. Korhonen MH, Brunstein J, Haario H, Katnikiv A, Rescaldani R, Hedmna K. A new method with general diagnostic utility for the calculation of immunoglobulin avidity. Clin Diagn Lab Immunol 1999;6:724–728.
23. Pollard AJ, Levin M. Production of low-avidity antibody after infection with serogroup B meningococci. Lancet 2000;356:265–2066.
24. Vermont CL, van Dijken HH, de Groot R, van den Dobbelsteen GP. Porin A-specific antibody avidity in patients who are convalescing from meningococcal disease. Pediatr Res 2005;58: 149–152.