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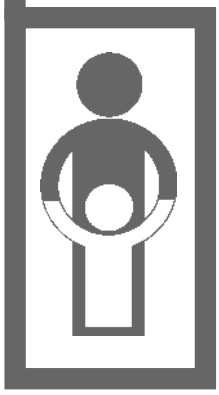
Standardization and validation of serological assays for the evaluation of immune responses to *Neisseria meningitidis* serogroup A/C vaccines

Geneva, 8-9 March 1999



DEPARTMENT OF VACCINES AND BIOLOGICALS

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World Health Organization
Vaccines and Biologicals
CH-1211 Geneva 27, Switzerland
• *Fax:* +22 791 4193/4192 • *E-mail:* vaccines@who.ch •

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1. Background, objectives and format of the meeting

WHO's conservative estimate for the meningococcal meningitis disease burden is of 300 000 to 350 000 cases and more than 30 000 deaths a year. All countries suffer from endemic meningococcal disease, which primarily affects children under the age of five years, the annual attack rate being around 1 to 3 per 100 000 population. In addition, some countries, predominantly but not exclusively in the developing world, suffer from occasional or even regular epidemics of meningitis. These, frequently caused by group A strains, involve attack rates ranging from about 10 per 100 000 to 400-800 per 100 000 annually. Although strains of serogroups B and C are most prevalent during endemic periods they have also been responsible for outbreaks and epidemics on a reduced scale.

The next few years will be extremely important for the development and licensing of new meningococcal conjugate vaccines. Seroroup C meningococcal conjugate vaccines are likely to be the first vaccines licensed for use in infants where efficacy will not be measured directly but inferred from immunogenicity data. A similar approach may be used for licensing and formulating recommendations for new conjugate vaccines being developed for prevention serogroup A meningococcal disease. WHO is also reassessing the optimal use of serogroup A meningococcal polysaccharide vaccines for epidemic prevention or routine immunization in the meningitis belt countries of Africa. It is therefore becoming critical to elucidate whether currently existing serological assays provide appropriate data for future decisions on licensing and recommendations for use.

The laboratory assays that have been classically used to evaluate and compare immune responses to meningococcal vaccines fall into two basic groups: antibody-binding and functional. Antibody-binding assays (ELISAs) measure both functional and non-functional antibodies. Typically, they measure antibodies of lower avidity than is the case with functional assays, unless modified to preferentially measure high-avidity antibodies. Low-avidity antibodies are often not functional. Functional assays (e.g. animal models, opsonophagocytic assay, serum bactericidal assay (SBA)), tend to measure only high-avidity antibodies and are a better measure of protective efficacy. If a high correlation between these two assay groups is observed (e.g. between ELISA and SBA), then either assay should be acceptable for evaluating and comparing immune responses to meningococcal vaccines.

Controversy over optimal laboratory methods for ELISA and SBA has to be resolved before laboratory correlates can be recommended for vaccine licensure. With regard to the IgG ELISA for serogroup A/C meningococcus, concern exists that the standard CDC assay measures both high-avidity and low-avidity antibodies and that results from sera with predominantly low-avidity antibodies do not accurately predict the

observed SBA titre. An expert panel should therefore consider whether the current standard ELISA is satisfactory for assessing vaccine immunogenicity and what role, if any, the modified ELISA may have in improving assessment.

With regard to the serogroup A/C SBA, there is debate about what is the best complement source for the standard assay. Rabbit complement tends to give higher SBA titres than human complement. However, human complement may be too insensitive in some cases and its standardization may be difficult because of human donor variability. The group was asked to consider whether the convenience of using rabbit complement for the bactericidal assay was justified or whether a strong preference should be given to the adoption of human complement as the standard, and was asked to make recommendations on this matter.

In addition, WHO requirements for meningococcal polysaccharide vaccines established in the 1970s and modified in 1981 may need to be revised (*WHO Technical Report* Series, 1976, No. 594, Requirements for meningococcal polysaccharide vaccine).

Thus the main focus of the meeting was as follows:

- To assess whether the existing standard serological assays provide sufficiently unambiguous data to permit decisions for licensing and public health recommendations of meningococcal serogroup A/C vaccines.
- To determine what additional studies, if any, should be conducted in order to resolve the remaining problems associated with the current assays or to develop improved assays.

The format of the meeting was as follows:

- The first day was devoted to the delivery and discussion of the presentations (see Annex 1).
- A preliminary document reflecting the discussions of the first day and identifying a set of unresolved issues was drafted during an evening session involving George Carlone, Peter Densen, Keith Cartwright, Ian Feavers, Carl Frasch, Dan Granoff and Luis Jódar. These issues were discussed on the second day. Three members of the drafting group prepared a set of recommendations, and these were discussed and agreed by all the participants (listed in Annex 2).

2. Pathogenesis, mechanisms of host immunity and epidemiology of meningococcal disease

2.1 Pathogenesis of meningococcal infection and mechanisms of host immunity[1]

2.1.1 Pathogenesis of meningococcal infection

A. Meningococcal structural components implicated in invasion:

Pili: the attachment

1) Capsular antigen
   (a) Serogroup A
   (b) Serogroup C
2) Subcapsular antigens
   (a) Outer membrane proteins
   (b) Lipooligosaccharide

B. Acquisition

1) Close human contact
2) Disease occurs in minority of persons who acquire organism
3) Time from acquisition to disease is relatively short

C. Invasion – result of ligand receptor interaction

1) Organism – genetic mechanisms regulating antigenic variation; ligands
2) Host – genetic regulation of expression of cell membrane structures; receptors
   (a) Constitutive expression
   (b) Modulated expression - inflammation
      - Viral infection
      - Smoke

1 Prepared by Dr Peter Densen.

D. Disease

1) Endemic – 1 case per 100 000 population; predominantly a disease of childhood

2) Epidemic – >10 cases per 100 000 population; affects all ages with relative increase in teenagers

3) Two major forms

(a) Meningitis predominant

- Approximately 60% of cases
- Organisms largely confined to meninges and cerebrospinal fluid
- Focal inflammatory response
- Mortality < 10%

(b) Bacteraemia predominant (meningococcaemia)

- Approximately 30% of cases
- Can be fulminant
- Intravascular organism load and endotoxin concentration very high
- Systemic inflammatory response (shock)
- Mortality approximately15-20%

2.1.2 Mechanisms of host immunity

A. How does the host protect itself?

1) Antibody - most important

(a) What is the evidence?

- Age-specific incidence,10-15 cases per 100 000 population, in children < 2 years of age, mirrors host inability to produce antibody to (capsular) polysaccharides
- Pre-infection serum from adults who develop meningococcal disease lacks antibody to the infecting strain
- Meningococcal polysaccharide vaccines protect against infection
- Antibodies to capsular polysaccharide protect patients with immune defects predisposing to meningococcal infection

(b) Natural immunity – subcapsular cross-reactive antigens

- Meningococcal carriage – non-invasive strains
- Antigenically related organisms

(c) Acquired immunity – serogroup-specific in addition to subcapsular antigens

2) Complement – immune effector for antibody response

(a) What is the evidence?

- Deficient individuals have approximately1000 x risk of developing meningococcal disease
- Risk is greatest for persons who are unable to assemble the membrane attack complex (terminal complement component deficient)

(b) Implications for primary mode of meningococcal elimination/clearance

(c) Species specificity caveats

3) Phagocytic cells – potential effector for antibody/complement acting in concert

(a) What is the evidence?

- Excess representation of persons with PMN FcγRIIa among patients with meningococcal disease (antibody-receptor mismatch)
- Slight increase in risk of meningococcal disease in patients with reticuloendothelial dysfunction
- No apparent increase in meningococcal risk in patients with PMN defects

(b) Implications for primary mode of meningococcal elimination/clearance

B. Implications for vaccine trials

1) Vaccine – high-level response with immunological memory

(a) Conjugated – shift from TI to TD response

(b) CpG

2) Choice of assay

(a) Functional

- Bactericidal – apparently physiological
  - Complement source
  - Inoculum
  - Growth phase
- opsonophagocytic
  - PMN source and harvesting
  - Standardization for Fcγ receptors?

(b) Non-functional

- ELISA
  - Antigen-specific antibody

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2.2 Global epidemiology of serogroup A/C meningococcal disease and its control[2]

2.2.1 Epidemiology

In most of the developed world, meningococcal disease occurs at an endemic rate of 1-3 cases per 100 000 total population per year. As much as half of this disease may be attributable to serogroup B, which is not preventable with the polysaccharide vaccines at present available. Among the other major serogroups, serogroup C is the most common cause of meningococcal disease. Recently, numerous focal outbreaks of group C disease have been reported. They have received much public attention, partly because of the high case fatality rate (5-15%), the tendency for children of school age to be affected, and the occurrence of severe sequelae, including the amputation of limbs and mental retardation. The response to these outbreaks has included mass immunization with polysaccharide vaccine and increased development of serogroup C conjugate vaccines.

In the developing world the disease pattern is markedly different. In sub-Saharan Africa, epidemics of serogroup A disease with rates of up to 1000 to 2000 cases per 100 000 persons are of frequent occurrence. In 1996 the largest outbreak ever recorded, in which there were over 200 000 cases, struck central and western regions of sub-Saharan Africa. The number of cases decreased during the next two years but early in 1999 hundreds of cases were reported from western Sudan, indicating another substantial outbreak.

In the Eastern Mediterranean Region there is substantial concern over meningococcal disease. Outbreaks occur infrequently but a large one at the Haj in 1987 and subsequent smaller outbreaks have led to sustained vigilance. Several countries now routinely immunize children against the disease.

In the Western Pacific and South-East Asia Regions, frequent large outbreaks of serogroup A disease occurred in China and Mongolia, and the disease occasionally reached Nepal and India. However, in the last 20 years the disease rate has declined in China, and outbreaks have not occurred in Nepal or India since the mid-1980s. Large outbreaks of serogroup A disease occurred in Mongolia in the mid-1990s.

2 Prepared by Jay Wenger.

2.2.2 Vaccines and prevention

A single dose of the currently available polysaccharide vaccine gives short-term protection against the disease in older children and adults. However, the duration of protection is poor in young children and unclear in adults. Routine vaccination coverage in the regions most affected by epidemic disease is poor (i.e. 30-50% coverage with a single dose of measles vaccine in many countries in the meningitis belt). The most commonly used strategy for both A and C disease is surveillance aimed at early detection of outbreaks, and rapid implementation of emergency immunization. Other prevention options include preventive mass immunization (in areas suspected of being affected by outbreaks), routine administration of multiple doses in the first five years of life, and routine administration of a single dose to children entering school for the first time. Preventive immunization has been tried in countries bordering outbreak areas but its effectiveness is unclear, partly because the paths of outbreaks are not certain.

A vaccine providing long-lasting protection after an infant series would be the optimal prevention tool. The current generation of polysaccharide conjugate vaccines may meet this need, but studies in progress will have to be completed before we can be certain. If these vaccines are effective, industry must be assured of a reasonably large market. There is definite interest in seroroup C conjugate vaccines in North America and Europe. Although it is unclear which countries would adopt routine serogroup C conjugate or A/C conjugate immunization, the potential market (in terms of children born in countries wealthy enough to support purchase) in this area includes over 7.4 million neonates a year (Spain and the United Kingdom, 1.1 million; Belgium, France, Germany, and the Netherlands, 1.7 million; Denmark, Finland, Norway, and Sweden, 300 000; Canada and the USA, 4.3 million). In the Eastern Mediterranean Region, 0.85 million children are born annually in the Gulf States, which could also support introduction, and 2.3 million are born each year in countries already routinely using the polysaccharide A/C vaccine (including Egypt, Libya, and Syria,). Thus about 10 million children are born each year in countries which either already routinely use a meningococcal polysaccharide vaccine or could support conjugate vaccines at what will probably be market prices. Other Eastern Mediterranean countries bordering the meningitis belt account for an additional 1.7 million births a year.

About 7.1 million children are born each year in the sub-Saharan meningitis belt. They represent the largest single group of at-risk children where a relatively expensive immunization programme will be difficult to achieve. However, there is great concern about meningitis in these countries; coupled with the substantial market noted above, this may help to promote the development and use of the new vaccines wherever they are needed.

2.3 Discussion

The key features of the clinical disease and the pathogenesis of meningococcal disease were summarized. Structures of the organism such as capsular polysaccharide, pili, outer membrane proteins and lipooligosaccharide (LOS) play a crucial role in the ability of the organism to colonize and invade the host. Insights into the features of the host defence which are important in providing protection against meningococcal disease can be gained from individuals with immunodeficiencies causing a predisposition to meningococcal infection.

Risk factors such as smoking and viral infections were discussed. With regard to splenectomy as a risk factor for acquiring meningococcal disease, it was argued that in asplenic patients the phagocytic and antibody-producing roles of the spleen are lost and there is a lifelong susceptibility to infection. Although it is hard to prove benefit from preventive strategies, patients are likely to benefit from immunization with pneumococcal, *Haemophilus influenzae* type B and meningococcal vaccine before splenectomy.

Evidence suggesting that humoral antibodies were important in preventing meningococcal disease in humans dates back to studies by Kelmer et al. in 1918 (*J. immunol*., 3: 157) and Heist et al. in 1922 (*J. immunol* ., 7: 1). In 1969 the seminal work of Goldschneider et al. (*J. exp. med*., 129: 1307) showed that the incidence of meningococcal disease correlates inversely with the level of serum bactericidal antibody against meningococcus. Meningococcal polysaccharide vaccines offer protection against infection; age-specific incidence mirrors the host's inability to produce antipolysaccharide antibodies; and antibody to capsular polysaccharide can protect immunodeficient patients who are predisposed to meningococcal infection. Similarly there is convincing evidence that complement-mediated lysis is principally responsible for the elimination of organisms from the infected host, in that complement-deficient individuals have a greater risk of developing meningococcal disease, particularly those who are unable to assemble the membrane attack complex. The overrepresentation of people with PMN FcγRIIa receptors or with reticuloendothelial dysfunction among patients with meningococcal disease suggests that phagocytic cells may act together with antibodies and complement in the clearance of meningococci from the host.

Knowledge of the critical features of the human immune system informs decisions concerning vaccine design and the choice of serological assays. Reference was made to the potential for outbreaks of meningococcal disease in populations with elevated levels of circulating meningococcal IgA which can block serum bactericidal antibody. Although this was demonstrated by Griffiss et al. (*J. infect. dis.*, 1977, 136: 733-739) in military recruits, its relevance for the general population is questionable.

An overview of the global epidemiology of meningococcal disease was presented, with particular emphasis on disease occurring outside Europe and North America. The use of meningococcal polysaccharide vaccines for the prevention and control of epidemics in sub-Saharan Africa was discussed. The current approach to the early detection of meningococcal epidemics uses population-based weekly incidence thresholds. For countries in the meningitis belt, a rate of 15 cases per 100 000 per week in a given area, averaged over two consecutive weeks, appears to be a sensitive and specific predictor of epidemic disease. When this rate is identified an investigation

team should be sent to the affected area in order to confirm the etiology of the disease and, if appropriate, initiate an emergency response, including case management during the epidemic and mass vaccination of the entire population if vaccine supplies and administrative support are adequate. If resources are limited it may be necessary to restrict vaccination to the age groups most at risk. Alternative strategies have been suggested, whereby use of four doses of meningococcal group A vaccine is administered during the first five years of life (at 3, 7 and 24 months and > 5 years). Polysaccharide vaccines are being given to children when they first enter school in Egypt, Lebanon, and Syria. A number of countries, mostly in the Eastern Mediterranean Region, have adopted routine use of the polysaccharide vaccines in some form. For example, Saudi Arabia vaccinates the whole population every three years and all residents in the holy areas of Mecca and Medina annually. Rates of meningococcal disease in China have been declining steadily but it is far from clear whether this is related to the widespread use of polysaccharide vaccine. Valuable information on safety, duration of protection and antibody responses could be gathered from these countries. In this context the CDC is planning a case-control study in Egyptian schoolchildren.

3. Serum bactericidal assays (SBAs) and ELISAs as serological assays for the evaluation of immune responses to serogroup A/C meningococcal vaccines

3.1 Current assays revisited: scientific concerns and practical issues[3]

The next few years will be extremely important for the development and licensing of new meningococcal conjugate vaccines. Assessments of efficacy can rely on the results of immunogenicity studies, without the need for formal demonstration of vaccine efficacy, because complement-mediated bactericidal antibody correlates with protection against meningococcal disease (reviewed in Frasch) (1). Thus there is a well-accepted serological surrogate of vaccine efficacy which can be used as a basis for licensure the new conjugate vaccines by national regulatory agencies, for furthering research on new conjugate vaccines, and for assessing the efficacy of plain meningococcal A polysaccharide vaccines used in various vaccination schedules, age groups or populations. However, as described below, important concerns remain on the quality of the data being generated by the current standardized meningococcal serological assays. Do the results have a scientifically valid bridge to the previous seroepidemiological studies (Goldschneider et al. study in 1969) establishing bactericidal activity as a surrogate of efficacy? If not, are the discrepancies so great as to limit our ability to draw clear clinical inferences from the data, and to formulate reliable public health recommendations?

Two serological assays are in use as the principal means of measuring serum antibody responses to meningococcal vaccines. The ELISA, an antigen-binding assay, provides a good assessment of immune responses to vaccination but only an indirect measure of protective immunity. The reason is that qualitative differences, e.g. in avidity, may exist among antibodies which may be undetected by the ELISA but which can drastically affect the ability of antibodies to confer protection (2). A second assay, complement-mediated bacteriolysis, is therefore required to provide a measurement of antibody functional activity.

Both the ELISA and the meningococcal bactericidal assays have been considered in international workshops on the development of standardized laboratory protocols. These protocols were subsequently assessed in multicentre collaborative studies (3,4,5). The results demonstrated that different laboratories can obtain comparable results. However, as described below, the relationship between the antibody titres measured by these assays and protection against disease remains controversial.

3 Prepared by Dr D Granoff.

3.1.1 Bactericidal assay

The bactericidal assay measures the ability of serum antibody to kill meningococci in the presence of complement. Typically, test sera are heat-inactivated to eliminate endogenous complement activity. Different dilutions of test sera are then mixed with bacteria and a fixed concentration of exogenous complement, and incubated for 30 to 60 minutes. The bactericidal titre is expressed as the dilution of test sera resulting in 50% or 90% killing of the bacteria, compared to colony-forming units present at time 0.

Many factors can influence the results of measurement of meningococcal bactericidal activity. These include choice of the bacterial test strain, growth conditions of the bacteria, and the source of exogenous complement (human sera, or sera from a heterologous species such as the rabbit). The ideal exogenous complement is human serum from a patient with untreated agammaglobulinaemia and normal complement activity, since with this source the risk is negligible of introducing antibodies that can affect the measurement of bactericidal activity in the test sera.

Because of the widespread use of gammaglobulin treatment, however, only small amounts of agammaglobulinaemic serum from untreated patients are available for this purpose. An alternative exogenous complement source is therefore needed. The original studies in the 1960s demonstrating the correlation between serum bactericidal activity and protection from meningococcal disease used normal human serum as the complement source, and this lacked intrinsic bactericidal activity (6,7). This option was discarded during the workshop deliberations on the development of a standard bactericidal assay protocol against serogroup A and C strains, because it was expected that different laboratories would encounter difficulty in finding and standardizing reliable sources of complement from normal human serum. Furthermore, it was argued that infant rabbit complement gave results comparable to those obtained with human complement, and that rabbit serum could be prepared commercially, pooled and provided to different laboratories in a uniform and reproducible manner.

Zollinger and Mandrell first reported that the use of complement from a heterologous species could lead to falsely high serum meningococcal bactericidal antibody titres to serogroup B strains (8). Recent studies indicate that this also occurs with serogroup A or C strains. When meningococcal C bactericidal titres were measured in sera of infants from the United Kingdom given three doses of a meningococcal A/C conjugate vaccine, an eight-fold higher geometric mean titre was measured when rabbit serum was used as the complement source than when human serum was used (Borrow et. al., "Induction of immunological memory in UK infants by a meningococcal A/C conjugate vaccine", Abstract 157, 11th International Pathogenic *Neisseria* Conference, Nice, France, 1-6 November 1998). In adults primed with meningococcal A/C conjugate vaccine and boosted four years later with plain meningococcal polysaccharide vaccine the geometric mean bactericidal titre to meningococcal A measured with rabbit complement was over 70 times higher than that measured with human complement (Table 1).

Table 1: Meningococcal A bactericidal antibody responses of adults primed with meningococcal A/C conjugate vaccine four years earlier and boosted with plain polysaccharide vaccine*

	1/titer with human complement		1/titer with infant rabbit complement		
Subject	**Pre-booster**	**Post-booster**	**Pre-booster**	**Post-booster**	**Ratio post**
1	<4	540	<4	3248	6
2	<4	<4	<4	87	43
3	10	103	4	464	5
4	<4	41	16	444	11
5	<4	8	16	142	18
6	21	53	24	338	6
7	<4	92	<4	147	1.5
8	54	874	>64	2930	3
9	<4	23	<4	158	7
10	<4	8	<4	243	30
Geometric mean	4.1	44	6.3	369	8

* Healthy adult subjects were given a 1 mg dose of meningococcal polysaccharide vaccine as a booster 4 years after Meningococcal A/C conjugate vaccine priming (9).

Furthermore, there were examples of individual adult sera with bactericidal titres >1:4000 when measured with rabbit complement and undetectable titres (< 1:8) when measured with human complement (subjects 120-015 and 120-032 in Table 1). These results raise serious questions as to the clinical relevance of the presence of high meningococcal bactericidal antibody responses when measured with rabbit complement. It is vital to consider this matter as WHO reassesses the use of plain meningococcal A polysaccharide vaccine in infants and children in Africa and fosters research and development in improved conjugate vaccines for the prevention of disease caused by serogroup A and C strains.

In conclusion, the bactericidal assay provides an immunological surrogate of clinical protection but the results can be dramatically affected by assay methods, particularly the choice of human or rabbit complement. The strongest scientific link would be achieved if one could duplicate the Goldschneider assay method, which uses human complement, or demonstrate that alternative assays give comparable results.

3.1.2 ELISA

The bactericidal titre, if performed properly, provides a good surrogate of protective immunity. However, the bactericidal assay is a bioassay and therefore gives results of greater variability than those of well-controlled antigen-binding assays such as the ELISA. Also, bioassays tend to be less sensitive for the detection of antibodies and much more labour-intensive than antigen-binding assays. It is therefore highly useful to have an ELISA to measure total or isotype-specific serum antibody responses in a large number of vaccinated subjects. ELISA results provide an accurate assessment of vaccine immunogenicity and also provide information on the type of antibody response that helps distinguish plain polysaccharide from conjugated polysaccharide antigens (i.e. IgG vs. IgM; or IgG2 vs. IgG1 subclass). The ELISA therefore becomes the primary assay for assessing immunogenicity. The more variable and more labour-intensive bactericidal assay is reserved for assaying subsets of serum samples in order to confirm whether the anticapsular antibodies being measured in the ELISA are functionally active and likely to confer protection. Used in this way, the ELISA is particularly useful for comparing (bridging) antibody responses to meningococcal vaccination in different age groups or different populations, or for comparing different vaccines or assessing manufacturing consistency of different lots of vaccine. There are parallels to the use of these two assays with those used to measure antibody responses to *Haemophilus influenzae* type b (Hib) vaccines. For Hib, the Farr (an antigen-binding assay) was the primary measure of total antibody response to vaccination. Furthermore, minimal levels of serum antibody concentration were established that predicted protection from invasive Hib disease (0.15 µg/ml for short-term protection, 1.0 µg/ml for long-term protection). The bactericidal assay for Hib was used only in subsets of sera to confirm that the antibodies measured by the Farr assay had functional activity against the bacteria.

The current standard ELISA method for measuring serum antibodies to meningococcal A or C polysaccharides is both sensitive and reproducible (3,4). However, the results correlate poorly with bactericidal titres in assessments of heterogeneous populations of serum anticapsular antibodies. This is typically observed in young children given plain polysaccharide vaccine (10) or a single dose of conjugate vaccine (13). Heterogeneity of the antibody population may also be observed in randomized trials if one or more vaccines elicit, on average, higher avidity antibodies than the others (11,12).

Several recently reported meningococcal vaccine trials provide telling examples of poor correlation of ELISA antibody concentrations with bactericidal titres. Campagne et al. reported the antimeningococcal C antibody responses of infants in Niger who were immunized at 6, 10 and 14 weeks of age with either the meningococcal A/C conjugate vaccine or a licensed meningococcal A/C polysaccharide vaccine. After three doses of vaccine the geometric mean antibody concentrations measured by ELISA were virtually identical in the two groups. In contrast the bactericidal antibody responses of the infants given the conjugate vaccine were 30 times higher than in the polysaccharide group. Similar assay-related differences were observed by Lieberman et al. in a randomized study in toddlers in the USA who were vaccinated with the meningococcal A/C conjugate vaccine or a licensed polysaccharide vaccine (12). The ELISA indicated that the conjugate vaccine group had only a twofold higher serum antimeningococcal C anticapsular antibody

response after two doses of vaccine than the group that received the polysaccharide vaccine. In contrast, the geometric mean bactericidal titre was over 100 times higher in the conjugate vaccine group than in the polysaccharide group.

As noted above, the main purposes of the data generated by the ELISA are to provide a serological bridge between different immunogenicity studies and to compare the immunogenicity of different vaccines or batches of vaccine. Given the large differences in the bactericidal responses in the studies cited above, and the failure of the corresponding serum antibody responses measured by the standardized ELISA to distinguish between the vaccine groups, substantial concerns arise over the general value of the data being generated by the ELISA.

3.1.3 The relevance of high-avidity antibodies: the modified ELISA

Recently, a modified ELISA has been described which uses assay conditions primarily favouring the detection of higher-avidity anticapsular antibodies (13).

On a weight basis, higher-avidity antibodies are more effective than lower-avidity antibodies. Protection against disease consequently depends on both antibody concentration and avidity. Studies on serum antibodies to pneumococcal serotypes 6B and 23F indicate the importance of avidity in antibody effector functions and protection (14).

Figure 1: Effect of Antibody Avidity on Protection of Mice from Lethal Serotype 6B Bacteremia

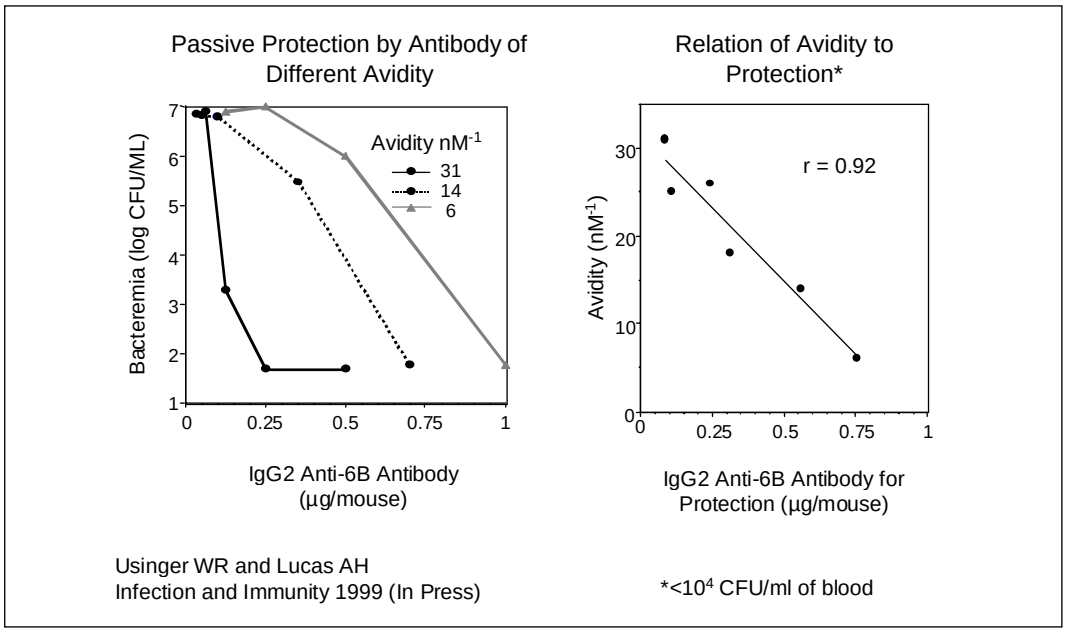
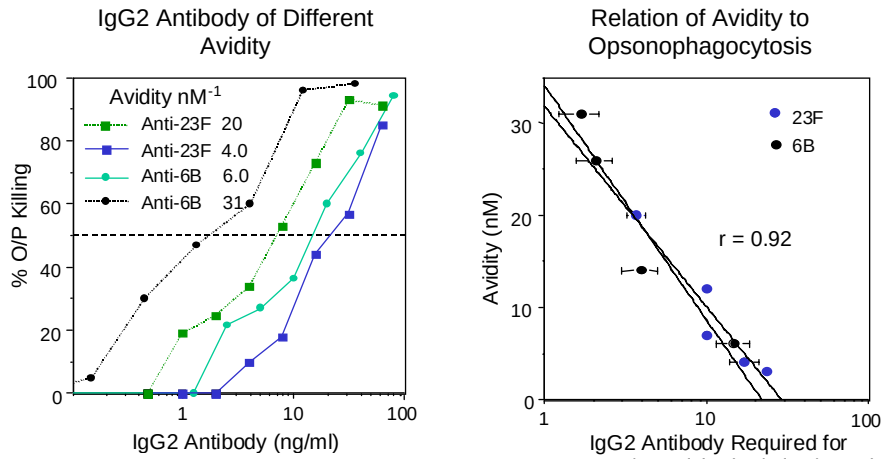


Figure 2: Effect of Antibody Avidity on Opsonophagocytosis of Serotype 6B and 23F Pneumococci



Usinger WR and Lucas AH
Infection and Immunity 1999 (In Press)

When assessing heterogeneous meningococcal C anticapsular antibody populations, such as those elicited by a conjugate or polysaccharide vaccine in toddlers, the results of this modified ELISA correlated better with bactericidal antibody titres than those obtained with the standardized ELISA (Fig 6 and Table 2).

Figure 3: Relationship between concentrations of IgG antibody to meningococcal C polysaccharide and bactericidal activity titers measured in serum samples obtained from toddlers 1 month after administration of a second dose of meningococcal polysaccharide vaccine or meningococcal C conjugate vaccine13

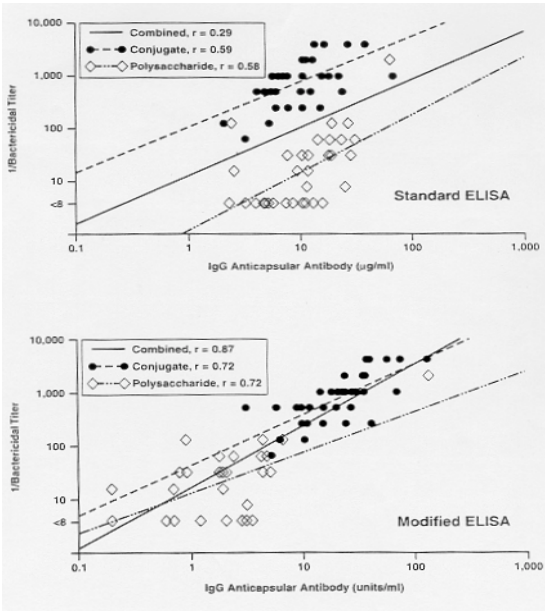


Table 2: Serum antibody concentrations of toddlers vaccinated with meningococcal C conjugate vaccine or meningococcal polysaccharide vaccine, as assessed by different assays*

	Geometric mean			Probability†	
	Pre-	**Post-1**	**Post-2**	**Post-1**	**Post-2**
IgG anticapsular antibody					
Standard ELISA, µg/ml					
– Polysaccharide vaccine	0.12	7.0	9.7	.11	.81
– Conjugate vaccine	0.18	5.1	9.3		
Modified ELISA, units/ml					
– Polysaccharide vaccine	0.22	1.0	1.2	<.001	<.001
– Conjugate vaccine	0.20	4.8	21.0		
Bactericidal antibodies, 1/titre					
– Polysaccharide vaccine	4.2	14	16	<.001	<.001
– Conjugate vaccine	4.4	74	761		

* Serum samples were selected from toddlers showing a wide range of IgG antibody responses to vaccination. There were 35 subjects in each group. (Granoff et al. (13))

† Comparing respective geometric mean antibody concentrations or titres elicited by the two vaccines.

Differences in antibody avidity assume greatest importance *in vivo* when serum antibody concentrations are limiting (after primary vaccination or when serum antibody concentrations have declined).

3.1.4 Conclusions

Meningococcal C conjugate vaccines will be licensed in the near future. These vaccines will be the first to be licensed for use in infants where vaccine efficacy will not be measured directly but will be inferred from immunogenicity data. Furthermore, recommendations on the number of doses to be given and the ages at which vaccination should be performed will be inferred solely from immunogenicity results. A similar approach will be used for licensing and formulating recommendations for new conjugate vaccines being developed for the prevention of meningococcal serogroup A, Y and W135 strains, and for re-evaluating the role of meningococcal A polysaccharide vaccine in certain areas. It is important to consider whether the existing standardized serological assays provide sufficiently unambiguous data to permit reliable decisions for licensing and public health recommendations. Will there be scientific distrust of the antibody titres being measured, leading to controversy and uncertainty about the efficacy of these vaccines and the wisdom of different recommended schedules?

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3.2 ELISA and bactericidal assays: how did we get where we are?[4]

Neisseria meningitidis serogroup A and C polysaccharide vaccines were first shown to be immunogenic in humans by Gotschlich et al. (1). These vaccines were developed at the Walter Reed Army Institute of Research and tested in military recruits (1); both haemagglutinating and bactericidal antibodies were elicited by these vaccines. Antibody responses to these capsular polysaccharide vaccines have been measured by various serological methods (1,2,3,4,5,6): capillary precipitation, haemagglutination, immunofluorescence, radial immunodiffusion, latex agglutination, opsonizing antibody, complement-fixing antibody, bactericidal antibody, radioimmunoassay (RIA), and enzyme-linked immunosorbent assay (ELISA). Meningococcal polysaccharide vaccines were licensed using data obtained by RIA and bactericidal assays. Of the quantitative procedures reported, only RIA and ELISA accurately measure capsular polysaccharide antibody levels with the serum volumes typically obtained from young children, and a serum bactericidal assay (SBA) has historically (1,2) been used to measure functional antibody titres. Both of these serological surrogates, and possibly others, will probably be used to license the new generation of meningococcal vaccines without the need for a classical efficacy trial. However, questions still remain as to the exact laboratory protocols necessary to accurately predict clinical protection.

3.2.1 Enzyme-linked immunosorbent assay (ELISA)

The earlier assays (RIA and ELISA) were designed to measure total antibody induced by natural infection or by polysaccharide vaccines; these responses were predominantly T-cell-independent and elicited IgG, IgM and IgA antibodies. With the development of protein conjugate vaccines and the ability to induce immunological memory, only IgG antibodies (presumably of high avidity) are measured by the current ELISA, since this is the predominant antibody class elicited after multiple doses of T-cell-dependent protein conjugate vaccines.

Because it is simpler and safer to perform than the RIA, the ELISA is preferred for routine measurement of immunoglobulin class and subclass concentrations and is the method of choice for a standard assay. Unlike serum bactericidal assays, opsonophagocytic assays or animal models, the ELISA is not a functional assay since both functional and non-functional antibodies (antibodies that bind but have no functional antibody activity) are measured. An ELISA that has a high correlation with standardized functional antibody assays is needed so that it can be used as a surrogate of protective immunity, if the licensing of new vaccines is going to be based predominantly on immunogenicity rather than clinical efficacy.

The results of two recent multilaboratory studies demonstrated that one can obtain reproducible measurements of serogroup A and C anticapsular antibody concentrations by a standardized ELISA (4,7). However, in some instances the ability to extrapolate from measurements of anticapsular antibody concentrations in serum to bactericidal antibody titres (functional antibody activity) appears to be limited (8). For example, in infants, toddlers and even in some adults who receive

4 Prepared by George Carlone and Susan Malanska.

meningococcal plain polysaccharide vaccine, one can detect high serum antibody responses to meningococcal polysaccharide by the ELISA in the presence of low or undetectable bactericidal antibody (8,9). The most likely explanation is that administration of the plain polysaccharide vaccine elicits principally low-avidity anticapsular antibodies, which are detected by the ELISA but appear to be less active in the bactericidal assay. These results are problematic if the IgG ELISA antibody responses are being compared after one or two doses of plain polysaccharide or protein conjugate vaccine; a single dose of either vaccine can elicit low-avidity antibodies, particularly in infants and toddlers (8,10).

For clinically meaningful results it is important to choose assay conditions permitting the assessment of functionally active antibodies that are likely to confer protection. To this end a modified IgG ELISA was developed (8) which favours the detection of primarily high-avidity antibodies. By measuring such antibodies selectively the modified assay gives results that correlate more closely with those of the bactericidal activity assay than with those obtained with the standard ELISA. The use of the modified ELISA appears to be most important if the immune responses to one or two doses of polysaccharide and protein conjugate vaccine are being compared (12); responses to a third or booster dose are similar using either assay. A confounding issue associated with the modified ELISA is that concentration units (µg/ml) are not appropriate with this protocol. This may present practical problems in the interpretation of results and may cause difficulty in linking to historical data.

3.2.2 Reference sera

Two reference sera, designated ECG and PB-2, have been widely used to estimate total meningococcal anticapsular antibody levels; the protective level of meningococcal serogroup A and C antibodies has been estimated by RIA to be 1-2 µg/ml of total antibodies (11).

ECG was prepared in 1969 from a single adult donor who had been immunized with a monovalent meningococcal serogroup A polysaccharide vaccine and subsequently with a monovalent serogroup C polysaccharide vaccine (12). Total meningococcal serogroup A and C anticapsular weight-based antibody units (µg/ml) were assigned by quantitative precipitation assay. PB-2 was prepared in 1987 from a single adult donor who had been immunized with the meningococcal quadrivalent vaccine (serogroups A, C, Y and W-135); arbitrary antibody units per millilitre were assigned.

In 1992 (13) a new standard meningococcal reference serum designated CDC1992 was prepared to replace reference sera ECG and PB-2. CDC1992 was prepared from a pool of 14 adult volunteers who underwent plasmapheresis following vaccination with a single dose of a *Neisseria meningitidis* quadrivalent polysaccharide vaccine (total and class-specific antibody concentrations in µg/ml were assigned). Twelve prevaccination and postvaccination quality control sera were similarly prepared. Antibody concentrations in reference sera ECG and PB-2 were compared to CDC1992 in order to provide a historical link to previous studies. These reference sera were produced from adults immunized with the polysaccharide vaccine, whereas the current need is to measure antibodies in infant sera elicited by protein conjugate vaccines. In practice it appears that CDC1992 is a suitable reference standard to use with the standard or modified IgG ELISA and SBA.

3.2.3 Serum bactericidal assay

The role of circulating antibodies and complement in protection against meningococcal disease was demonstrated in the 1900s (1) and was recently reviewed (6). Serum bactericidal antibody activity has been shown to correlate strongly with immunity to meningococcal disease (1,12). An inverse correlation was observed between the age-related incidence of disease and the age-specific prevalence of complement-dependent serum bactericidal activity (1). The induction of complement-dependent bactericidal antibodies following vaccination with meningococcal polysaccharide or protein conjugate vaccines is regarded as acceptable evidence of the potential efficacy of these vaccines (2).

In 1976 the WHO Expert Committee on Biological Standardization recommended an SBA to satisfy the requirements for production and release of meningococcal polysaccharide vaccine (5). These SBA requirements have also been used to support vaccine licensing. In order for a vaccine to be acceptable for licensing, over 90% of immunized adult subjects must have a fourfold rise (increase of 2 dilutions) in the SBA titre when tested against target strains by the specified SBA (5). A fourfold or greater rise in this titre is currently used to estimate the potential efficacy of meningococcal vaccines during field trials, as well as to determine seroconversion after immunization with the currently licensed polysaccharide vaccine or protein conjugate vaccines (9). Numerous procedures have been used to evaluate meningococcal serum bactericidal activity (9). They differ in various ways from the SBA recommended by WHO. Some of these methods were published prior to the WHO recommendations; however, some researchers continue to use and reference these earlier versions of SBAs. In most cases these assays are being used without an apparent comparison to the procedure recommended by WHO. A standard protocol should be adopted for the comparison and evaluation of new and developing meningococcal vaccines. Interlaboratory comparisons, such as will be necessary for conjugate vaccine licensing, are virtually impossible to interpret unless reagents and target strains are shared among laboratories using similar procedures. The use of non-standardized SBAs to measure functional activities of potential vaccines may lead to overestimation or underestimation of potential vaccine efficacy and possibly to premature release or delay of field trials and licensing (9).

The complement source used in the SBA appears to be of critical importance. Unlike the reported serogroup B SBAs that use human complement, the WHO recommended procedure for serogroups A and C involves the use of baby rabbit serum. A normal human complement source would seem to be desirable, but large volumes of a suitable human source are not available or readily obtainable in practice; furthermore, agammaglobulinaemic sera are unavailable in large volumes as a complement source.

Significant differences in antibody titres have been observed when SBA titres have been compared after using baby rabbit and human serum as complement sources: human complement tends to give lower titres (8,9). The original assays that associated bactericidal antibody with protection used human complement sources from individuals after natural infection (1), whereas vaccine licensing was supported by using SBA titres obtained from baby rabbit complement with sera before and after vaccination.

A recent multilaboratory study standardized the serogroup A and C SBA and compared it to the recommended WHO procedure (9). The standardized assay and the WHO recommended assay differed only by selection of the target strains, their growth and the final well volume. The modified assay will facilitate interlaboratory comparisons of the functional antibody produced in response to current or developing serogroup A and C meningococcal vaccines. However, a decision needs to be made regarding the complement source to be used in this protocol.

The SBA can be used to measure large numbers of sera in an immunogenicity study. Semi-automation can be achieved by the use of photographic plate readers and diluters. In fact, more sera can be assayed by SBA than by ELISA in the same time. The choice of assay for accessing vaccine immunogenicity is not, therefore, a limiting factor for large-scale immunogenicity studies or manufacturing consistency testing.

There are two standardized ELISAs that can reproducibly measure serum anticapsular antibody concentrations: a standard IgG ELISA that measures both high-avidity and low-avidity antibodies and a modified ELISA that measures predominantly high-avidity IgG antibodies. Results from these assays are, in general, not in good agreement when IgG antibody levels are compared after one or two doses of polysaccharide or protein conjugate vaccine. IgG ELISA levels after a single dose of either vaccine generally have a low correlation with SBA, presumably due to low-avidity antibodies. Correlation of the standard IgG ELISA and SBA is in good agreement after the primary vaccination series and after a booster dose. The use of a human complement source in the SBA tends to give much lower antibody titres than when baby rabbit complement is used; human complement, however, may be too insensitive for use in the assay even if available in sufficient volumes. The choice of complement source in the SBA needs to be determined.

A standard reference serum and 12 quality control sera are available for use in ELISA and SBA assays. The reference serum and quality control sera were prepared in adults using polysaccharide vaccine. However, it appears that this serum is suitable for determining antibody levels and SBA titres in infant and toddler sera from individuals vaccinated with the protein conjugate vaccine. Additional studies, however, may need to be done with these sera using SBA target strains and ELISA solid-phase polysaccharides that are acetylated and deacetylated.

In conclusion, questions still need to be answered and some research may need to be completed before we can confidently substitute these laboratory surrogates for clinical efficacy trials. Caution should be observed before clinical trials are abandoned and the selection or rejection of new meningococcal vaccine formulations is based predominately on results obtained from the current iteration of these laboratory protocols.

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3.3 Evaluation of human antibody response to serogroup C *Neisseria meningitidis* invasive infection using different immunological assays[5]

Meningococcal disease has been a significant public health problem in the city of Rio de Janeiro, Brazil, since the description of the first epidemic in 1921. Rio de Janeiro is the capital of Rio de Janeiro State, with a population of 6 million people. In 1988 the recorded incidence was 5 per100 000 and by 1995 it had increased to over 10 per 100 000. During the second half of the 1980s, serogroup B meningococcal disease emerged as a public health problem causing more than 90% of the confirmed cases. In 1993, serogroup C invasive infection started to replace serogroup B and in 1995 the serogroup C epidemic peaked.

An immunization campaign with the Cuban vaccine (VaMengoc B+C®) was carried out between May and July 1990 in an attempt to control the spread of serogroup B disease. About 1.6 million children aged 6 months to 9 years in the metropolitan area of Rio de Janeiro were vaccinated. A second mass campaign was instituted for persons up to 14 years of age between November 1994 and February 1995. As serogroup C disease continued to spread in the community another campaign with A + C polysaccharide vaccine (Mérieux®) was implemented for individuals aged 14 to 29 years.

Most studies on the immunology of meningococcal disease have focused on analysing the human immune response to meningococcal surface antigens after vaccination. There have been only a few studies on the immune response during the course of invasive meningococcal infection. Analysing the sera of patients may bring interesting insights into the natural history of meningococcal disease and may help in the evaluation and selection of candidate vaccines.

A critical review of current and alternative methods used to measure antibody response is also essential for the evaluation of a new generation of protein and conjugate vaccines against *Neisseria meningitidis*. Since the recurrence of meningococcal disease is rare in individuals with normal immune systems, the utilization of paired patient sera for assay evaluation will be a useful approach to selecting a surrogate assay method and studying the role of protective antibodies to different cell surface antigens.

3.3.1 Patients and methods

Study population

Patients admitted with a diagnosis of meningococcal disease and bacterial meningitis to the Meningococcal Disease Reference Centre (Instituto Estadual de Infectologia São Sebastião) in the city of Rio de Janeiro between April 1995 and April 1996 were enrolled in the study. Most patients had received preadmission antibiotic treatment. We conducted a prospective cohort study to investigate the antibody responses of patients with meningococcal disease according to their vaccination status, using different immunological methods. Cases of meningococcal disease (MD) and bacterial meningitis were defined by either the isolation of bacterium from blood or cerebrospinal fluid (CSF) or the detection of bacterial capsule antigens in CSF by the latex agglutination test.

5 Prepared by David E. Barroso, Lucimar G. Milagres, Maria C. Rebelo and Carl E. Frasch.

The medical charts of meningococcal disease and bacterial meningitis patients in the documentation section of the hospital were thoroughly reviewed. A patient was considered to have been immunized when the vaccination status (VaMengoc B+C®, 1994-1995 immunization campaign) was noted on the appropriate sheet of the medical chart.

Serum samples

The acute-phase serum was drawn within 24 hours after hospital admission and convalescent serum was obtained between 15 and 30 days (mean 21 days) after admission. Serum IgG concentrations (µg/ml) to *N. meningitidis* C polysaccharide were determined for all acute and convalescent sera using the Centers for Disease Control (Atlanta) standard ELISA assay (C polysaccharide mixed with methylated human serum albumin).

Preliminary results

Paired sera were collected from 101 patients during the study period; 35% had serogroup B disease (35/101), 40% had serogroup C disease (40/101), and 25% had bacterial meningitis caused by *Haemophilus influenzae* type b (n=18) or *Streptococcus pneumoniae* (n=8). The prevalent meningococcal phenotypes causing disease were B:4:P1.15, B:4:P1.7 and C:2b:P1.10.

The ages of the patients ranged from 4 months to 52 years (mean = 11; median = 6); 60% were males and 40% were females. Fifteen of the patients had been immunized with two doses of the Cuban vaccine. Nine of the 15 immunized patients had serogroup B meningococcal disease, 4 had serogroup C disease, and 2 had *H. influenzae* type b meningitis; their ages ranged from 5 months to 9 years. Two of the serogroup C meningococcal immunized patients were infants (< year old), one was 2 years old and the fourth was 4 years old. The mean age of the immunized serogroup B patients was 4 years and that of the serogroup C patients was 1.7 years.

The proportions of non-immunized patients with acute-phase IgG C polysaccharide antibody levels higher than 1 µg/ml were 28% (95% confidence interval: 14-45%) for serogroup C meningococcal patients and 27% (95% confidence interval: 12-48%) for serogroup B patients, compared to 8% (95% confidence interval: 1-7%) in the bacterial meningitis group ($P < 0.05$). Of the 13 immunized patients with meningococcal disease, 54% (95% confidence interval: 25-80%) showed an acute phase IgG antibody concentration to the C polysaccharide higher than 1µg/ml, compared to 27% (95% confidence interval: 17-40%) of the non-immunized meningococcal patients ($P = 0.06$). Twenty-nine percent (2/7) of vaccinees with 1µg/ml IgG C polysaccharide antibodies in their acute sera had a significant decrease (≥ fourfold) in antibody level (convalescent sera), compared to none (0/17) among non-vaccinees ($P < 0.05$).

Seventy-two percent (95% confidence interval: 55-86%) of non-immunized serogroup C meningococcal disease patients had at least a fourfold increase in antibody levels to the C polysaccharide in their convalescent sera, compared to none among the serogroup B disease patients and only one of the bacterial meningitis patients ($P < 0.01$; sensitivity 78%; specificity 98%; PV + 97%; PV - 86%).

However, two of the four vaccinees who developed serogroup C disease showed at least a fourfold increase in antibody levels during the convalescent phase ($P > 0.05$). The mean age of the 26 C non-immunized patients with a positive response was 17 years, compared to 13 years for those (n = 10) with no response. The proportion of patients ≤2 years old was 15% (95% confidence interval: 4-34%) and 40% (95% confidence interval: 12-74%), respectively for those with and without a positive response ($P = 0.1$). For the immunized serogroup C patients with and without a positive response the mean ages were 2.3 and 1.2 years respectively.

3.3.2 Preliminary conclusions

The human immune response to *N. meningitidis* invasive disease, as measured by the C polysaccharide ELISA, seems to be polysaccharide-specific, and no cross-reactivity was seen among serogroup B meningococcal disease and bacterial meningitis patients. Nevertheless, a proportion of both immunized and non-immunized patients with serogroup C disease did not show a significant increase in antibody during the convalescent phase and most of these were young children. Our data indicate that patients may not develop an adequate C polysaccharide immune response in early infancy. Immunization status was associated with a higher acute-phase antibody level and a decrease in antibody concentration during early convalescence.

Additional studies are being conducted as follows. (i) Standard ELISA with the addition of thyocyanate in the serum-diluting buffer (in order to measure high-avidity antibodies); (ii) modified ELISA (Granoff, DM et al., 1998) intended to measure high-avidity antibodies, in which the C polysaccharide is covalently bound to ADH; (iii) serum bactericidal assay (SBA) using a Brazilian group C *N. meningitidis* strain. The SBA, a functional assay, will provide the standard for this study and will be used for comparison with the other methods.

Table 3: Serum IgG concentrations (µg/ml) to *N. meningitidis* C polysaccharide in acute and convalescent sera of patients measured by standard ELISA, modified ELISA and modified ELISA mixed with methylated human serum albumin

	mHSA ELISA		**Modified ELISA**		**Modified mHSA ELISA**	
Clinical cases	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**
Serogroup **C** disease (n = 40)	1.48	11.29	1.37	6.61	1.08	2.73
Serogroup **B** disease (n = 35)	1.58	1.57	1.78	1.69	1.16	1.14
Bacterial meningitis (n = 26)	1.09	1.22	1.09	1.1	1	1
		***P*<0.05**				***P*<0.05**

Table 4: Proportion of non-immunized patients with at least a fourfold increase in IgG C polysaccharide antibodies, according to three different ELISA assays

Clinical cases	mHSA ELISA	Modified ELISA	Modified mHSA ELISA
Serogroup C disease (n = 36)	72% (95% CI: 55-86%)	50% (95% CI: 33-67%)	42% (95% CI: 26-59%)
Controls* (n = 52)	S = 72% (95% CI: 55-85%)	S = 50%	S = 41%
	E = 98% (95% CI: 88-99.9%)	E = 100%	E = 100%
	VP+ = 96% (95% CI: 79-99.8%)	VP+ = 100%	VP+ = 100%
	VP- = 84% (95% CI: 72-91%)	VP- = 74%	VP- = 71%
	2% (95% CI: 0-10%)	0% (95% CI: 0-7%)	0% (95% CI: 0% - 7%)
	(c2 = 46; *P* < 0.01)	(c2 = 30; *P* < 0.01)	(c2 = 23; *P* < 0.01)

* Serogroup B and bacterial meningitis patients.

3.4 Meningococcal serogroup A/C vaccine assay status: the United Kingdom experience[6]

The high incidence of meningococcal serogroup C disease in the UK led to the initiation of a Public Health Laboratory Service (PHLS) Vaccine Evaluation Programme. The overall purpose was to accelerate the introduction of meningococcal conjugate vaccines into the UK immunization programme. This Programme, initiated in 1994, commenced with phase II trials of candidate meningococcal serogroup C conjugate (MCC) vaccines under the UK 2-3-4-month schedule. Following encouraging results in the primary immunization trials, the Programme was extended for three years in order to provide the UK Department of Health with the necessary information to plan for the introduction of MCC vaccines into the UK immunization schedule. The UK Vaccine Evaluation Consortium (VEC), comprising the PHLS, the National Institute of Biological Standards and Control (NIBSC), the Centre for Applied Microbiological Research (CAMR) and the Institute of Child Health (ICH), is conducting this Programme.

Initial phase II trials in infants included three different MCC vaccines: a first-generation Chiron A/C CRM, two dose levels of the Wyeth-Lederle Vaccines and Paediatrics (WLVP) C CRM and a North American Vaccine (NAVA) C tetanus toxoid. Additional studies now under way include a direct comparison of three different MCC vaccines (a second generation Chiron C CRM, a WLVP C CRM and a NAVA C tetanus toxoid) in toddlers aged 2-3 years to assess whether a single dose of MCC vaccine is adequate for long-term immunity. A similar study is being

6 Prepared by Ray Borrow and the UK Vaccine Evaluation Consortium.

conducted in preschool cohorts (aged 3-4 years) and school-leaving cohorts (aged 15-16 years) with the objective of assessing whether prior, concurrent or simultaneous administration of existing diphtheria/tetanus-containing vaccines interferes with the reactogenicity and immunogenicity of MCC vaccines (and vice versa). In a further study, the possibility that exposure to meningococcal polysaccharide (MPS) vaccines may induce immunological hyporesponsiveness is being explored. This involves administering either two doses of MPS or one dose of MPS followed by a dose of MCC either 6 or 12 months after the first dose.

A large and valuable collection of sera has been provided by these vaccine trials, particularly where direct comparison of the three manufacturers' vaccines has been possible. To satisfy the UK Department of Health and the manufacturers, the vaccine recipients' sera were initially assayed by the serum bactericidal assay (SBA), using rabbit complement (C'), and by the ELISA as recommended by the CDC, Atlanta (1-4). These assays were found to be highly reproducible and easy to standardize between laboratories. Additional assays were then performed on the remaining sera. These assays included an ELISA for high-avidity antibodies (5), the use of human C' in place of rabbit C' in the SBA, and the avidity index as a surrogate marker for successful priming (6).

In order to compare assays for serogroup C the WLVP C CRM primary trial sera were investigated. Sera were assayed by SBA using rabbit and human C', while the ELISAs included the CDC assay for total Ig and IgG and the assay for high-avidity antibody. Depicted below are geometric mean concentrations of serogroup C-specific Ig, IgG and high-avidity IgG comparing three ELISA assays and SBA GMT measured by rabbit or human C'.

Figure 4: Geometric mean concentrations of serogroup C-specific Ig, IgG & high avidity IgG antibody in UK infants determined either by:

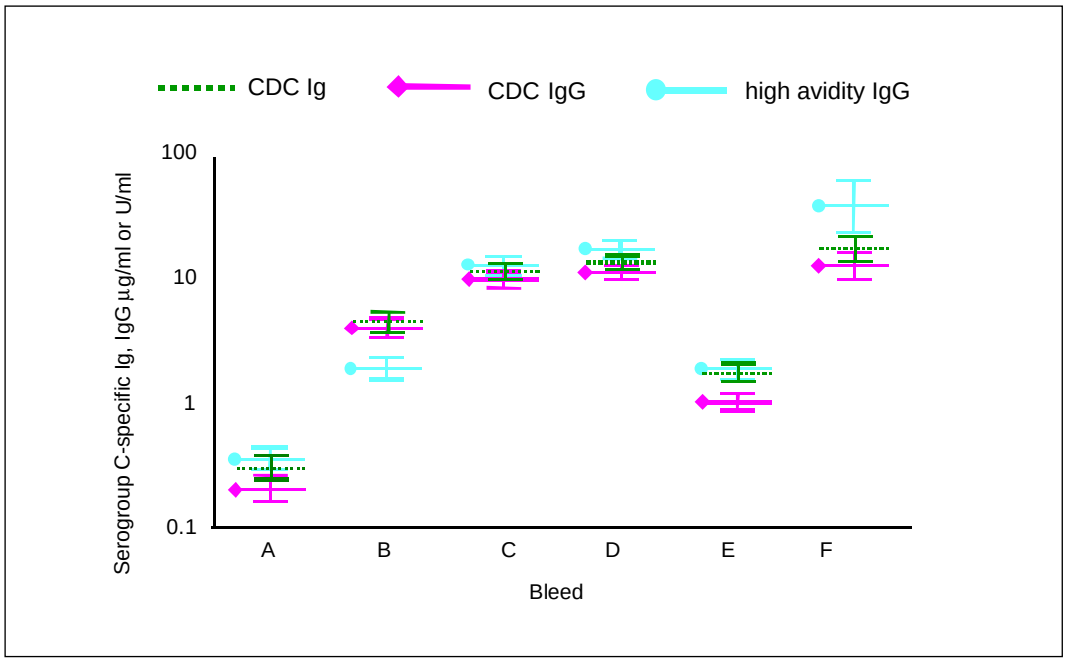
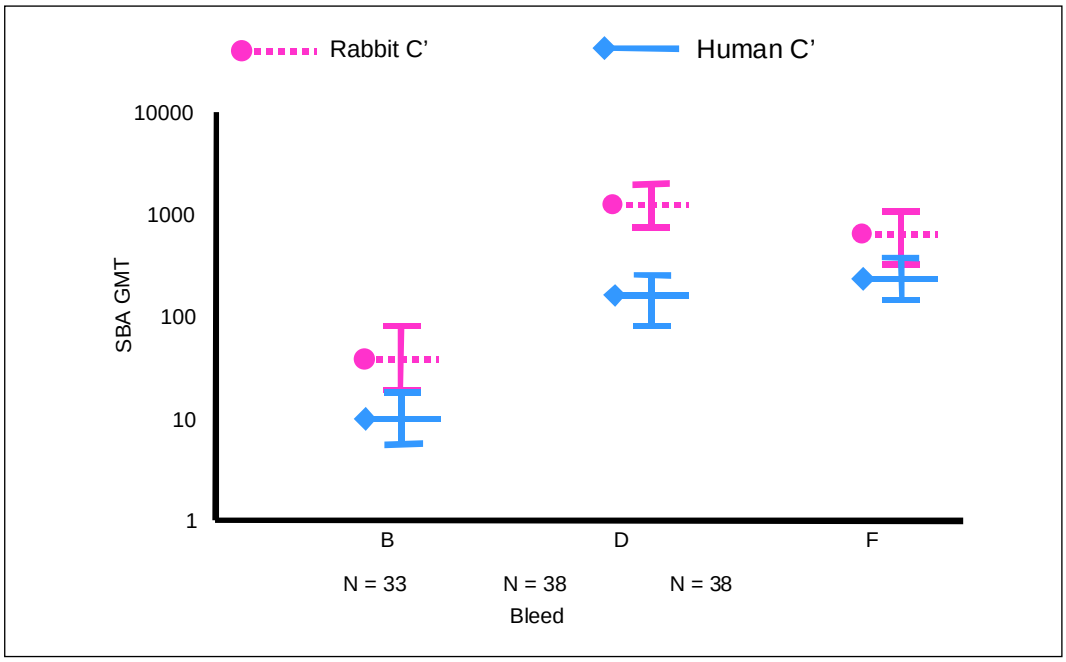


Figure 5: Serum bactericidal geometric mean titres determined either by:



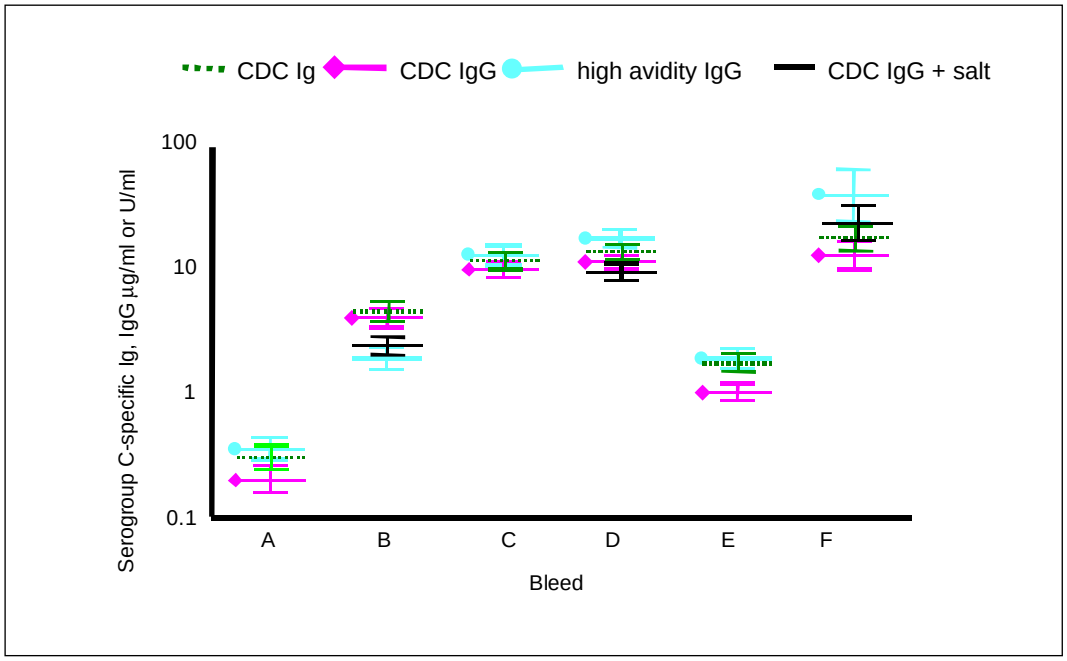
Correlations (r), when all bleeds were combined (i.e. prevaccination (A) and after one (B), two (C) and three doses (D) and prebooster (E) and postbooster (F)) between the SBA with rabbit C' and CDC Ig, CDC IgG and high avidity were 0.905, 0.915 and 0.916 respectively, while comparable correlates were obtained for the SBA with human C'. SBA titres were higher when rabbit C' was used than with human C'. After one dose, three doses and booster, there were 4.3-fold, 8.2-fold and 2.7-fold differences between geometric mean titres (GMTs) for SBA rabbit versus human C'. The differences were greater for high-titre sera. When regressions of SBA using human C' versus rabbit C' were plotted a regression of 0.9 was achieved, but when individual bleeds were examined separately the slopes were found to be 0.532 after one dose, 0.763 after three doses and 0.949 following booster. Therefore the differences in SBA assays are much less for high-avidity antibodies produced after polysaccharide boosting and almost approach equivalence.

An alternative way to analyse the differences between SBA for rabbit versus human C' is to look at the proportions of responders. Considering a responder as having an SBA titre of ≥1:8 for rabbit C' or ≥1:4 for human C' after one dose, 70.5% and 54.5% of infants were responders using rabbit and human C' respectively, while the proportions of responders were identical after three doses and booster for both C' sources (97.4%). For this MCC vaccine it appears that high-avidity antibodies are being induced after three doses or booster and that the type of SBA assay used is therefore less important. The typr of SBA is more relevant following one dose, when presumably low avidity-antibodies are being induced, as a difference is noted, underlined by the number of responders when considering the SBA. Studies in the 1960s suggested the protective titre for the SBA using human C' to be ≥1:4. Using the present data it was possible to calculate the equivalent titre for rabbit C' as ≥1:10. However, it must be borne in mind that this conclusion is drawn from data

obtained from recipients of MCC vaccination and might not be applicable to immunity resulting from disease, from vaccination with non-conjugated C polysaccharide, or from vaccination with protein and other non-polysaccharide antigens (relevant to protection against serogroup B disease).

The addition of 75 mM thiocyanate in either CDC or high-avidity assay (including the standard serum), as opposed to the CDC standardized protocol, lowers IgG levels after a single dose and raises IgG levels after multiple doses or boosting.

Figure 6: Geometric mean concentrations of serogroup C-specific Ig, IgG & high avidity IgG antibody in UK infants determined either by:



Further studies were performed on serogroup A assays, utilising sera from the first generation Chiron A/C conjugate vaccine. Infants received 0.25 ml meningococcal serogroup A/C conjugate vaccine and 0.25 ml aluminium hydroxide adjuvant.

Correlations between ELISA (total Ig) with rabbit C' and human C' were found to be 0.114 and 0.657 respectively. On comparing the SBA GMTs for rabbit C' versus human C', 1.6-fold, 6.8-fold and 35.0-fold differences were recorded following one dose, three doses and booster, the proportions of responders being 24%, 78.8% and 93.8% for rabbit C' and 19.1%, 52.2% and 74.1% for human C' respectively. The high-avidity assay will be applied to these sera.

We have also developed an assay for the assessment of antimeningococcal C antibody avidity for qualitative assessment of antibodies elicited by different MCC conjugate vaccines and for assessing how this relates to the bactericidal activity of antibodies induced by the different vaccines. The relative change in avidity over time will also be examined after primary immunization and before and after booster immunization, in order to evaluate whether antibody avidity can be used as a surrogate marker for priming for immunological memory as has been demonstrated for Hib conjugate vaccines (6).

When comparing different manufacturers' vaccines it is also essential to consider the antigen source and, in particular, whether the polysaccharide constituent is either acetylated or deacetylated. Sera from toddlers receiving a single dose of the Chiron, WLVP and NAVA MCC vaccines have shown a difference dependent on the acetylation status of the polysaccharide antigen. Antibody levels were found to be twofold higher when deacetylated antigen was utilized in the CDC ELISA assay as opposed to acetylated when studying deacetylated vaccines. The effect of deacetylation in the SBA assay will now be examined.

In summary, both the CDC ELISA and SBA assays can be very reproducible and easy to standardize. The use of the high-avidity ELISA instead of the CDC assay appears to be less important when considering conjugate vaccines alone as opposed to comparisons between polysaccharide and conjugate vaccines, as good correlation with SBA may be achieved with either. The effect of the high-avidity ELISA assay has yet to be investigated for serogroup A. SBA titres tend to differ markedly, depending on the C' source, especially where serogroup A SBAs are concerned

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3.5 Views from industry

3.5.1 Pasteur Mérieux Connaught[7]

Pasteur Mérieux Connaught (PMC) is developing a meningococcal conjugate vaccine comprising serogroups A and C polysaccharide which are covalently attached to diphtheria toxoid protein. During the course of this development, two clinical studies have been undertaken in collaboration with WHO, CDC and CERMES in Niamey, Niger. The first consisted of a dose-ranging study comparing the safety and immunogenicity of three separate formulations of the experimental bivalent A/C meningococcal conjugate vaccine to those of the licensed bivalent A/C polysaccharide vaccine and a *Haemophilus influenzae* type b conjugate vaccine (PRP-T). In the second, which is still in progress, one of the vaccine formulations from the first study is being evaluated for safety and immunogenicity following several vaccination schedules that can be applied to the EPI vaccination schedule.

In the first study, 180 infant subjects were randomized into five vaccine groups with 36 infants in each. Three groups received the experimental meningococcal A/C conjugate vaccine at 6, 10 and 14 weeks of age. The three formulations that were evaluated in this study contained 1, 4, or 16 (g of each serogroup polysaccharide per 0.5 ml dose; these vaccines were formulated into phosphate-buffered physiological saline. The fourth group received a placebo dose of physiological saline at 6 weeks of age followed by two doses of the licensed meningococcal A/C polysaccharide vaccine at 10 and 14 weeks of age. This vaccine consists of 50 mg of each serogroup polysaccharide formulated in physiological saline. The fifth group served as a control and received three doses of *Haemophilus influenzae* type b polysaccharide conjugated to tetanus toxoid protein, at 10 mg polysaccharide per dose formulated in tris-buffered physiological saline at 6, 10 and 14 weeks of age. At 18 weeks of age, the fifth group was offered a dose of meningococcal A/C polysaccharide vaccine. All five groups received DTP and OPV at 6, 10 and 14 weeks, and yellow fever vaccine and measles vaccine at 9 months of age. Blood specimens were taken from all of the subjects at 6 and 18 weeks of age. In addition, the first four groups were followed up at 11 months of age, a blood specimen being taken prior to the administration of a dose of meningococcal A/C polysaccharide vaccine. Blood specimens were taken one month following the dose of polysaccharide at 12 months of age.

7 Prepared by Robert P. Ryall, M. Bybel and C. Ethevenaux.

Table 5: Safety and immunogenicity of three doses of a *Neisseria meningitidis* A/C diphtheria conjugate vaccine in infants from Niger

wks	Age 6 wks (5-8 wks)	Age 10 wks (9-12 wks)	Age 14 wks (13-16 wks)	Age 18 mths (17-20 wks)	Age 9 12 mths	Age 11- mths	Age 13
EPI vaccines	DTPI, polio	DTP2, polio	DTP3, polio		Measles YF		
Groups 1,2,3: men. A/C conjugate n = 108 children (3 subgroups of 36 given 1, 4 and 16 mcg doses)	Men. D	Men. D	Men. D			Men. PS	
Group 4: men. A/C polysaccharide n = 35 children	Saline placebo	Men. PS	Men. PS			Men. PS	
Group 5: Hib PRP-T n = 37 children	PRP-T	PRP-T	PRP-T	Men PS			
Venipuncture (all groups)	X			X		X	X

Men. D: meningococcal A/C conjugate vaccine.
Men. PS: meningococcal A/C polysaccharide vaccine.
X: blood drawn.

The blood specimens taken in this study were analysed by ELISA and serum bactericidal assays that measured antibodies to both serogroup A and serogroup C polysaccharide. The methods used to measure the immune responses were developed by George Carlone's laboratory at the CDC. Both PMC and the CDC measured the serum bactericidal activity in the 6-week and 18-week blood specimens. A comparative analysis of the results obtained from both laboratories is shown below.

Table 6: Comparative serum bactericidal activity titre GMTs (CDC vs. PMC)

Serogroup	CDC		PMC	
	Pre	Post	Pre	Post
A	11.7	55.8	9.3	44.3
C	50.9	53.2	24.4	21.2

The ELISA assays were conducted by PMC. The CDC is planning to perform a subset of ELISA for interlaboratory comparison. PMC performed the serological assays, ELISA and serum bactericidal assays, using the CDC assay methods on the 11-month and 12-month blood specimens.

In the second study the 4 µg experimental meningococcal A/C conjugate vaccine formulation is being evaluated for safety and immunogenicity following a variety of immunization schedules that can fit into the EPI vaccination schedule. In this study, 618 infant subjects were randomized into one of six vaccine groups, each containing 103 infants. Group 1 received three doses of the experimental vaccine at 6, 10 and 14 weeks and a booster at 9 months of age. Group 2 received three doses of the experimental vaccine at 6, 10 and 14 weeks. Group 3 received one dose of the experimental vaccine at 14 weeks and a booster at 9 months. Group 4 received one dose of the experimental vaccine at 14 weeks of age. Group 5 received one dose of the experimental vaccine at 9 months. Group 6 received one dose of the licensed meningococcal bivalent A/C polysaccharide vaccine at 9 months of age. All the groups are scheduled to receive a dose of polysaccharide vaccine at 24 months of age. All participating infants will be simultaneously vaccinated with DTP and OPV at 6, 10 and 14 weeks, and with yellow fever vaccine and measles vaccine at 9 months as scheduled by the national EPI programme for infants in Niger. Groups 5 and 6 received *Haemophilus influenzae* type b conjugated to tetanus toxoid protein at 6, 10 and 14 weeks.

Blood specimens are taken from all groups at 18 weeks, and at 10, 24 and 25 months of age. Throat swabs are taken at 10 months and 24 months of age to assess nasopharyngeal carriage in this population.

Table 7: Immunogenicity and response to polysaccharide challenge following different schedules of a serogroup A/C diphtheria conjugate vaccine administered to infants in Niamey, Niger

		6 wks	10 wks	14 wks	18 wks	9 mths	10 mths	15 mths	24 mths	24 mths + 1 wk
Group	**Schedule**	**DTP OPV**	**DTP OPV**	**DTP OPV**		**YF Measles**	**OPV**	**DTP**		
1	2,3,4,9 m	Men.D	Men. D	Men. D	X	Men. D	X +swab		Men. PS X +swab	X
2	2,3,4 m	Men. D	Men. D	Men.D	X		X +swab		Men. PS X +swab	X
3	4m 9m			Men. D	X	Men. D	X +swab		Men. PS X +swab	X
4	4m			Men. D	X		X +swab		Men. PS X +swab	X
5	9m	Hib	Hib	Hib	X	Men. D	X +swab		Men. PS X +swab	X
6	2,3,4,9m	Hib	Hib	Hib	X	Men. PS	X +swab		Men. PS X +swab	X

X: blood drawn

Sample size calculations are focused on assuring that a sufficient number of infants will be available to meet the first study objective and to provide information at the 24 + 1 week visit on the capability to induce an anamnestic immune response to polysaccharide challenge following a primary series of conjugate or polysaccharide vaccine administered during the first year of life.

Enrolment of the 618 infants in this study is complete, and the 9-month follow-up will be completed in June 1999. The serological results from this study are not yet available.

3.5.2 SmithKline Beecham Biologicals[8]

Bactericidal assay

The bactericidal activity of animal and human sera are tested as previously described (1,2), with only slight differences. Serial twofold serum dilutions are incubated with log phase meningococci adjusted to 4 x 10^4 CFU/ml and incubated for 15 minutes at 37°C. Baby rabbit complement (Pel-freeze Biologicals, US) is added and incubation proceeds for 60 minutes. Aliquots of the mixture are spotted on to agar plates and incubated overnight at 37°C with 5% CO_2. Bactericidal titres are determined as the dilution giving 50% killing.

Table 8: Preliminary comparison between SBA developed at SmithKlineBeecham (SB) vs. the CDC (1997) SBA

Sample	SBA@SB	CDC 1997	
		Microscope	***ELISPOT***
7 pre	<8	<16	16
7 post	9300	>128 000	>128 000
16 post	600	>1024	>1024
12 post	4000	6400	7200
41 post	500	>1024	>1024

Anti-polysaccharide ELISA assay

Anti-polysaccharide antibody levels of animal sera are tested according to the CDC protocol (3) with only slight differences. Briefly, methylated BSA is coated at 5 (µg/ml in PBS on microplates for 2 hours at 37°C, followed by meningococcal A or C polysaccharide in PBS overnight at 4°C. Serial twofold serum dilutions are tested and reference sera are included. Specific anti-polysaccharide antibodies are detected after incubation with goat anti-mouse IgG antiserum. Antibody titres of individual sera are calculated by using the four-parameters logistic equation.

8 Prepared by Gaetane Metz, Matt Hohenboken and Jan Poolman.

Figure 7: Antibody affinity measured by mHSA

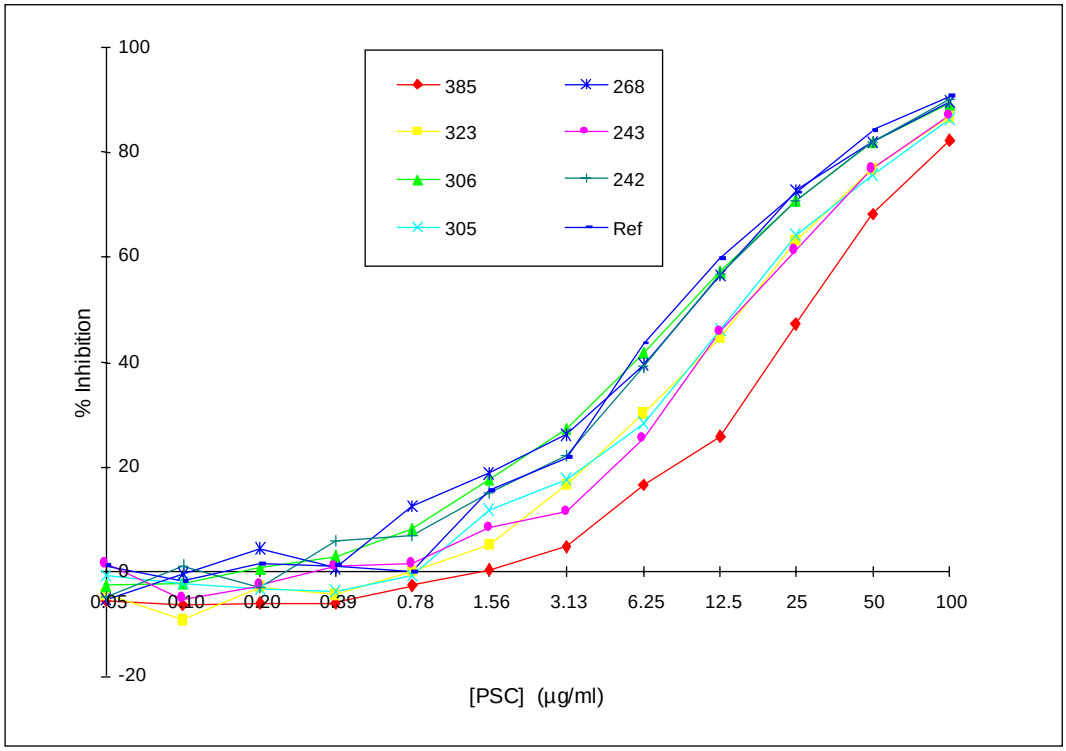
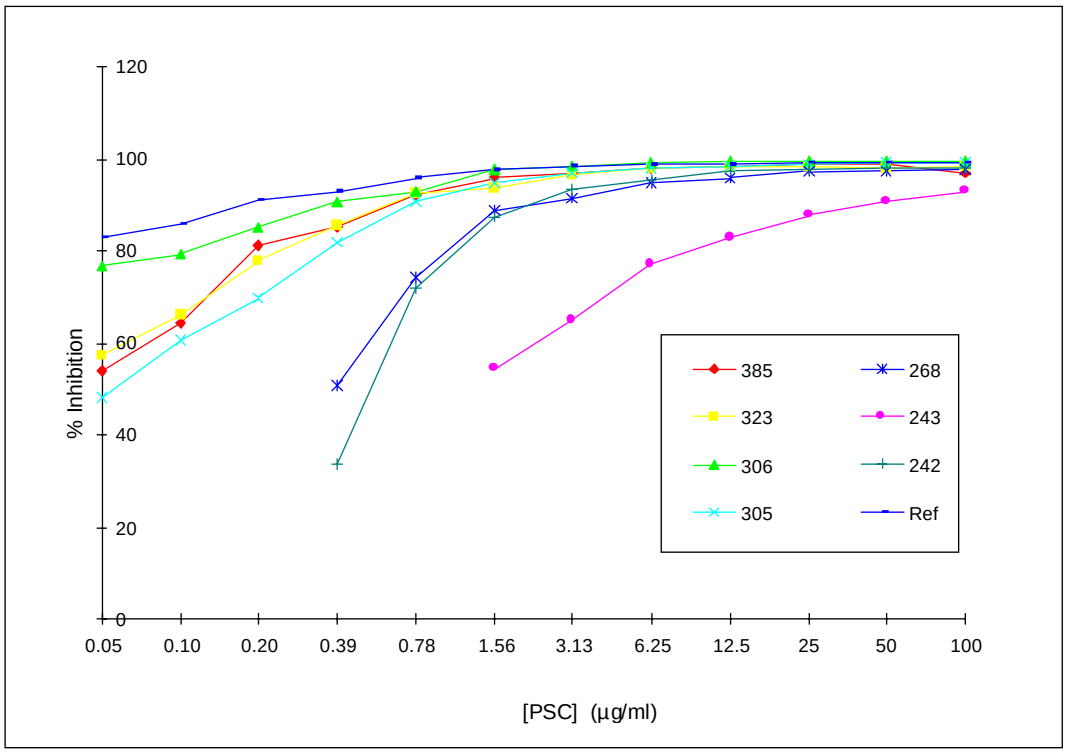


Figure 8: Antibody affinity measured by mBSA ELISA



Clinical data

A clinical trial included randomized administration of a single dose of either meningococcal conjugate vaccine or licensed polysaccharide bivalent vaccine (Mencevax AC®) to 50 healthy adults. All subjects had antibody detectable by both assays from sera collected one month after vaccination (≥1:8 bactericidal titre, or 0.300 mcg/ml), although up to one third were without detectable antibodies prior to vaccination. The correlation coefficient for comparison of the two assays in serodetectable samples is 0.81.

Despite the fact that the bactericidal titres and the IgG concentration correlate better after conjugate immunization, the Pearson correlation 95% confidence intervals indicate that this is not significant (Fig 9 and 10)

Figure 9: Adults, d28 Post Conjugate Immunisation

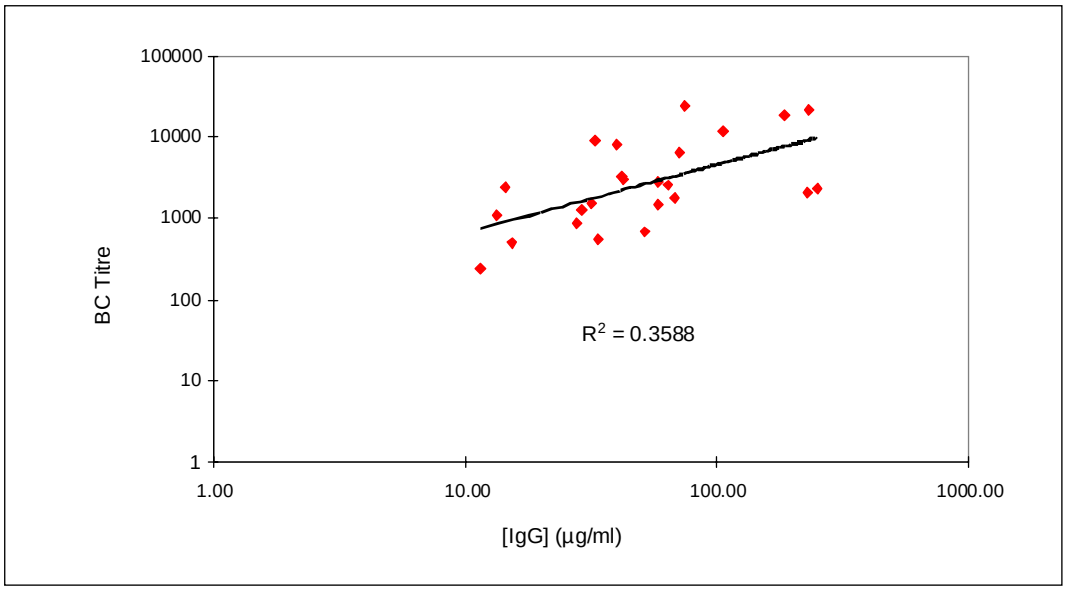
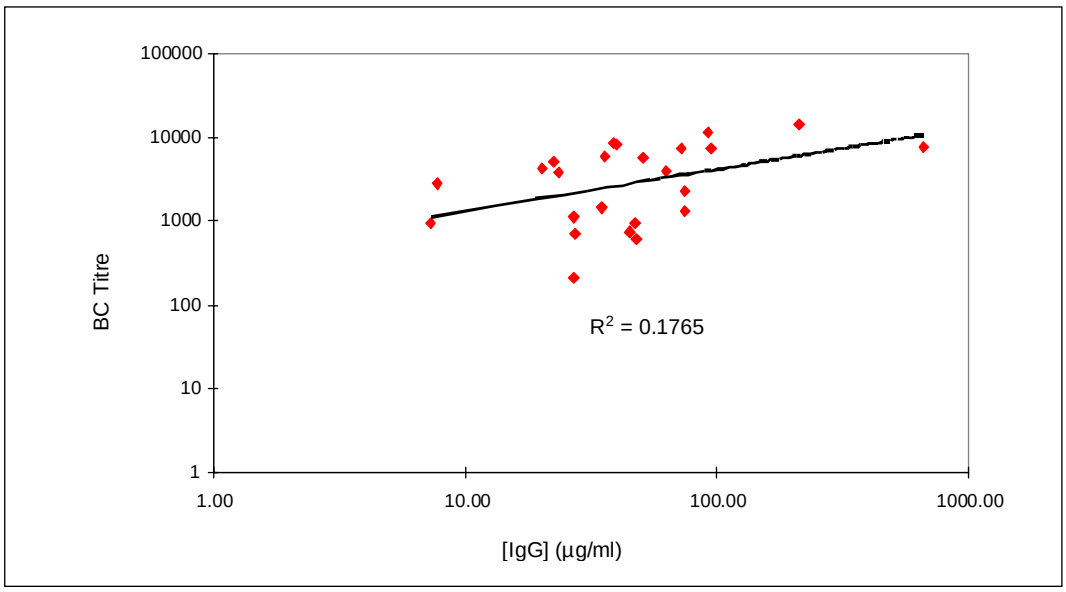


Figure 10: Adults, d28 Post Mencevax AC Immunisation



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3.5.3 North American Vaccines[9]

The group C meningococcal polysaccharide (GCMP) conjugate vaccine developed and manufactured at North American Vaccine (NAVA) was evaluated in the UK by the Institute of Child Health and Public Health Laboratory Service in Phase I (adults, 1 IM injection) and Phase II (infants, 3 IM injections, 2-3-4 months) clinical trials (1, 2). This vaccine consisted of the de-O-acetylated form of the polysaccharide (PS) coupled to tetanus toxoid (TT) by reductive amination (3). The de-O-acetylated form of GCMP was chosen for its greater immunogenicity as a conjugate when compared with its O-acetylated form in mice, which included higher bactericidal (BC) titers (4). Others had previously reported similar results in humans with PS vaccines (5, 6, 7). Each dose contained 10 µg PS, 15.5 µg TT, 0.5 mg aluminum hydroxide, and 0.01% thimerosal. Postimmune sera were sampled at 1 month after each injection.

The PS-specific IgG and IgM were measured at NAVA by ELISA (8), using the CDC 1992 reference serum (9) to standardize the IgG concentrations. For the coat antigen, de-O-acetylated GCMP was conjugated by reductive amination to human serum albumin (HSA) as an irrelevant carrier protein that would enable binding to the microtiter plate (8). These HSA conjugates were chosen as coat antigens for their ease of use, stability, and consistency.

Serum BC activity (SBA) was determined at NAVA using both infant rabbit sera and adult human sera as exogenous complement sources in order to assess the functional activity of the antibodies. The BC titer was defined as the reciprocal serum dilution that resulted in 50% killing (8).

Rabbit complement resulted in BC titers that averaged 4.4-fold higher than what was seen with human complement when assaying infant sera after 1, 2, or 3 injections with the GCMP-TT conjugate vaccine; all but 1 were < or = 12-fold higher (80/81). A strong correlation (r = 0.799, n = 81), however, was shown for the SBA when comparing rabbit with human complement. All subsequent SBA correlations reported here were performed with rabbit complement.

9 Presentation prepared by Peter C. Fusco, Esmé K. Farley, Max Kristiansen, and Francis Michon.

Figure 11: Rabbit versus Human Complement for Measuring Serum Bactericidal Activity in GCMP-TT Vaccinated Human Infants

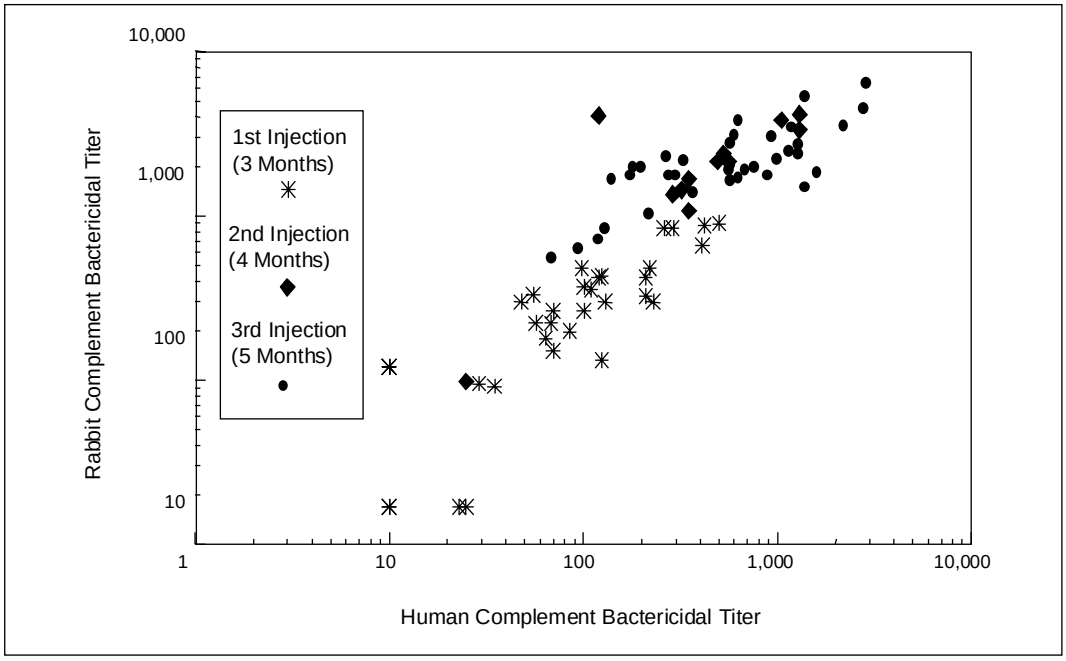


Figure 12: Polysaccharide-Specific IgG versus Bactericidal Activity for GCMP-TT/Adsorbed Conjugate Vaccine in Humans

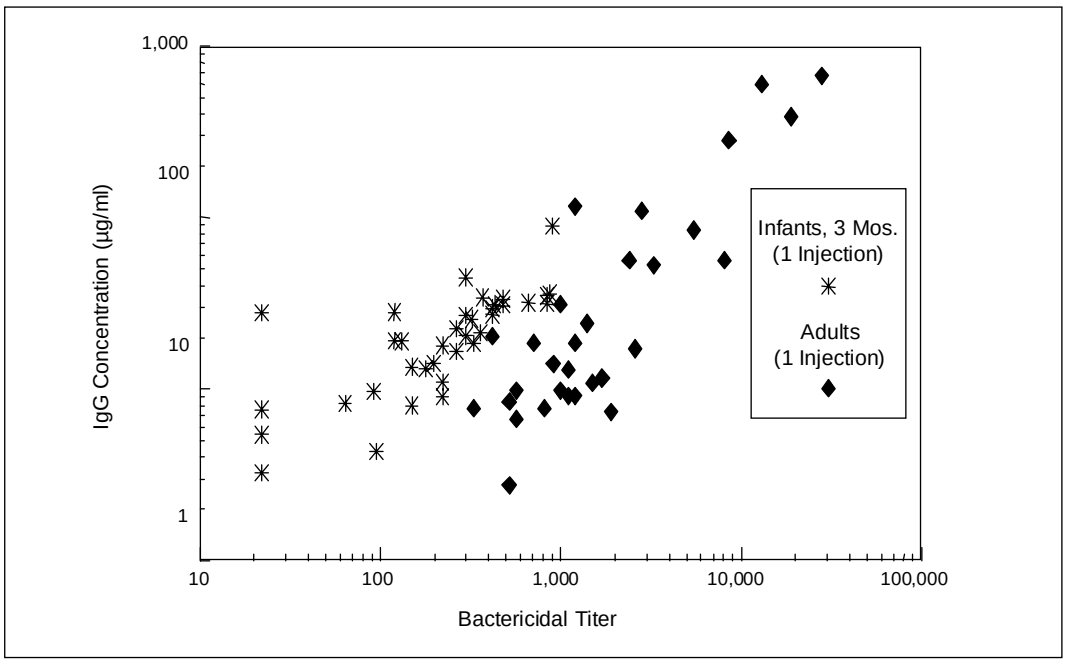
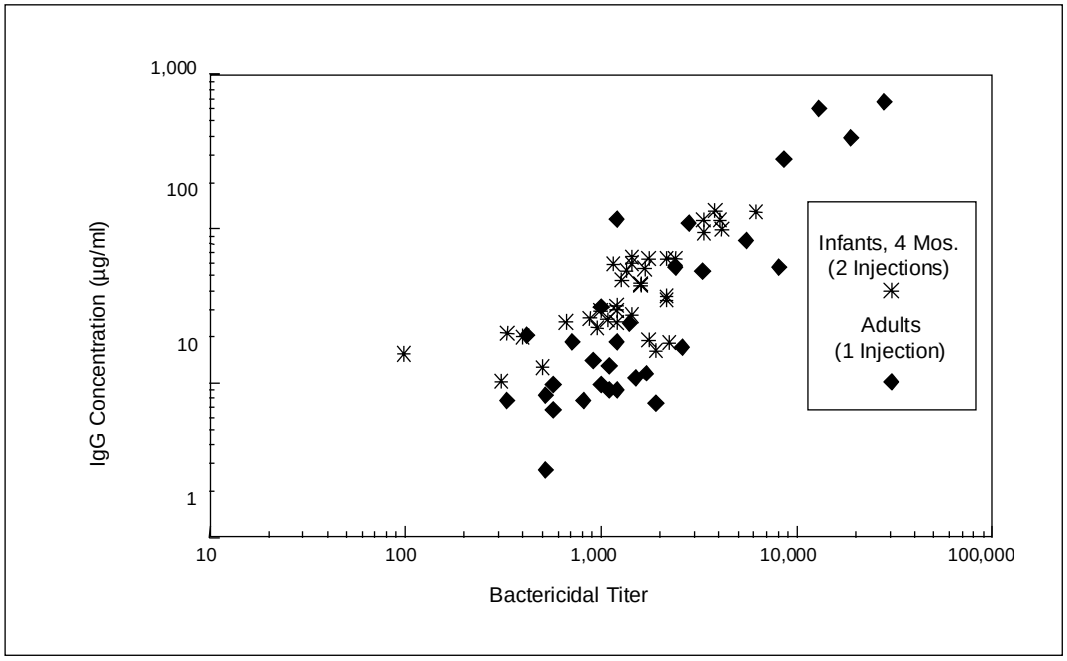


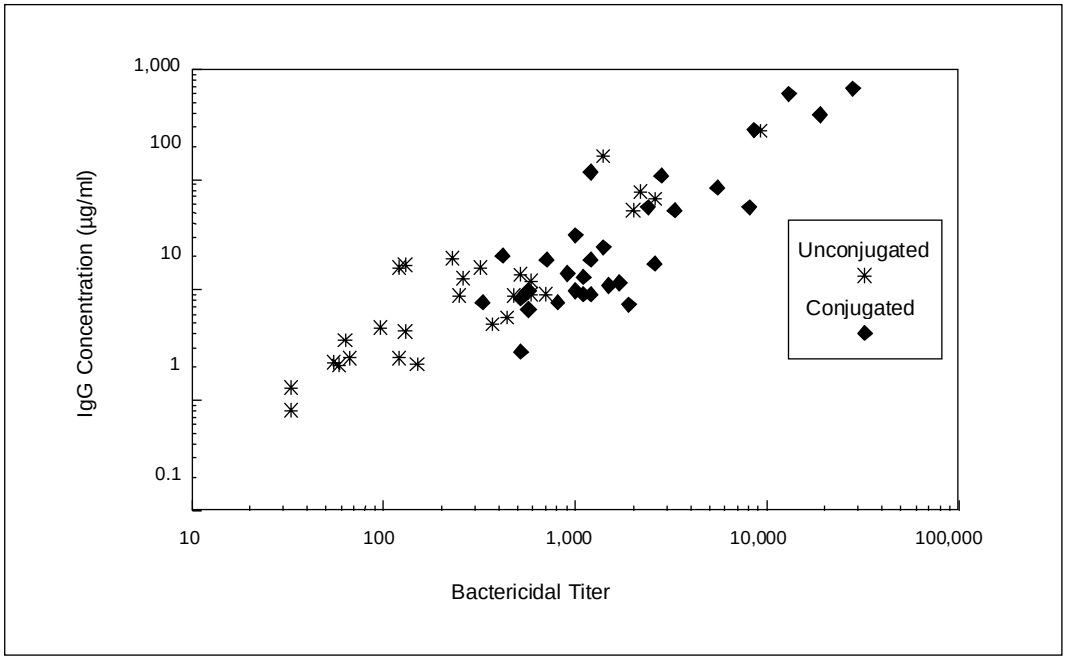
Figure 13: Polysaccharide-Specific IgG versus Bactericidal Activity for GCMP-TT/Adsorbed Conjugate Vaccine in Humans



Strong correlations were seen between the IgG concentration and the BC titer for adults after 1 injection ($r = 0.861$, $n = 30$) and infants ($n = 36$) after 1 ($r = 0.731$, Figure 2A), 2 ($r = 0.761$, Figure 2B), and 3 ($r = 0.723$, Figure 2C) injections. These correlations were well maintained even with inclusion of preimmune sera (e.g., adults: $r = 0.891$). Interestingly, the scatter plot of infant sera at 5 months (post 3rd injection) was essentially superimposable on that of the adult sera i.e., the graphic display of SBA per IgG was nearly identical for adults and infants ($r = 0.834$). However, there was a shift in response for the sera from the 1st (Figure 12) and 2nd (Figure 13) injections in infants toward lower BC titers per IgG compared to adults. No significant correlation was observed for IgM with SBA in the adults ($r = 0.100$). The infants, however, did show some correlation after the 1st injection ($r = 0.575$) that became weaker by the 3rd injection ($r = 0.331$).

Sera evaluated from 28 UK high school students one month after receiving a licensed meningococcal AC polysaccharide vaccine (ACVAX, SmithKline Beecham, UK) showed strong correlation between IgG and SBA ($r = 0.890$). This scatter plot had also fit the same correlation line as that of the adults receiving the NAVA conjugate vaccine ($r = 0.867$ when combined, Figure 14), although the IgG and SBA were both significantly reduced for the PS vaccine when compared to the conjugate vaccine ($p < 0.001$).

Figure 14: Polysaccharide-Specific IgG versus Bactericidal Activity for GCMP Vaccines in Human Adults after 1 Injection



In summary, strong correlations were routinely observed between IgG and SBA using rabbit complement for both adult and infant human sera. While assays with human complement did result in lower BC titers compared to rabbit complement, there appeared to be no advantage or necessity to choose human complement over rabbit complement.

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3.5.4 Wyeth Lederle Vaccines[10]

As new vaccines for *Neisseria meningitidis* serogroup C develop and are applied to younger populations, assessing vaccine immunogenicity with serological methods must be considered carefully to maximize efficiency while maintaining accuracy and usefulness. Two standard procedures currently exist for vaccine serology: the serum bactericidal assay (SBA) and the anticapsular specific enzyme-linked immunosorbent assay (ELISA). We have used these standard procedures to assess the immunogenicity of meningococcal group C polysaccharide conjugated to CRM197 (MnCC) or quadrivalent polysaccharide vaccine.

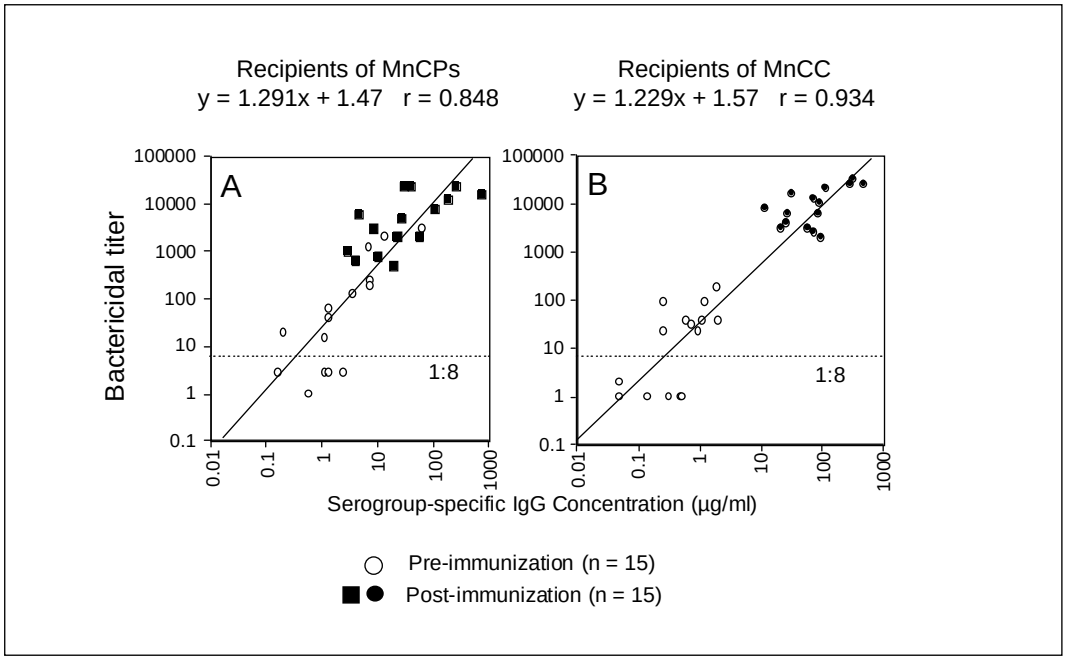
We have made minor modifications in the standard procedures, and these have improved our assay performance for large clinical trial evaluations while maintaining validation to the standard procedures. (1,2,3). For the SBA we use only freshly plate-grown bacteria and test our sera at a starting dilution of 1:2. For the ELISA we use medium binding plates, removed a separate blocking step, and high-quality antigen and pyrogen-free buffers and diluents.

ELISA technology has a clear advantage in large-scale vaccine population studies. However, we recognize that vaccine development for *N. meningitidis* serogroup C has, historically, relied heavily on functional immunity assessment using the SBA. The newer vaccines are targeted for infant populations and it becomes prohibitive to perform large serum-consuming bioassays on each child. Our goal has been to evaluate both assays during our vaccine development programme and to determine the appropriate situation for substituting the ELISA for the SBA.

Adults receiving either MnCC or quadrivalent polysaccharide vaccine were assessed by means of the ELISA and the SBA before immunization and a month after vaccination. Both vaccines induced rises in antibodies. When comparing the relationship between the ELISA IgG concentration and the SBA titre, a similar correlation was observed ($r = 0.93$ and $r = 0.85$ respectively).

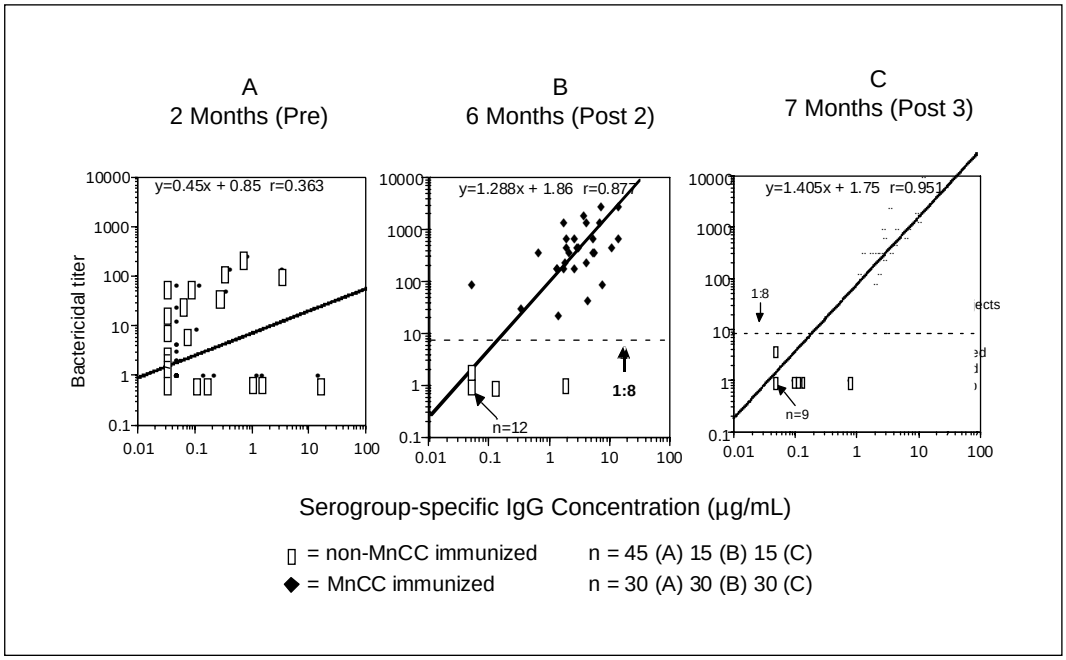
10 Prepared by Dace V. Madore, Daniel J. Sikkema, Ih Chang, Stephen W. Hildreth and Sally A. Quataert.

Figure 15: Relationship of ELISA IgG and SBA Activity in Adult Sera Pre- and Post-Immunization with MnCPs or MnCC



During vaccine evaluations in infant and toddler populations we have always compared the vaccine serology to that found in age-matched controls. Similar assessment of ELISA and SBA in children receiving MnCC has demonstrated significant rises in ELISA IgG concentrations and SBA titres. A notable problem with SBA measurements in sera of very young infants is that SBA activities can be high due to maternal antibodies or neonatal proteins and there may be specificity to other antigens than meningococcal group C polysaccharide.

Figure 16: Relationship of ELISA IgG and SBA Activity in Infant Sera Pre- and Post-Immunization with MnCC and with Age-Matched Controls

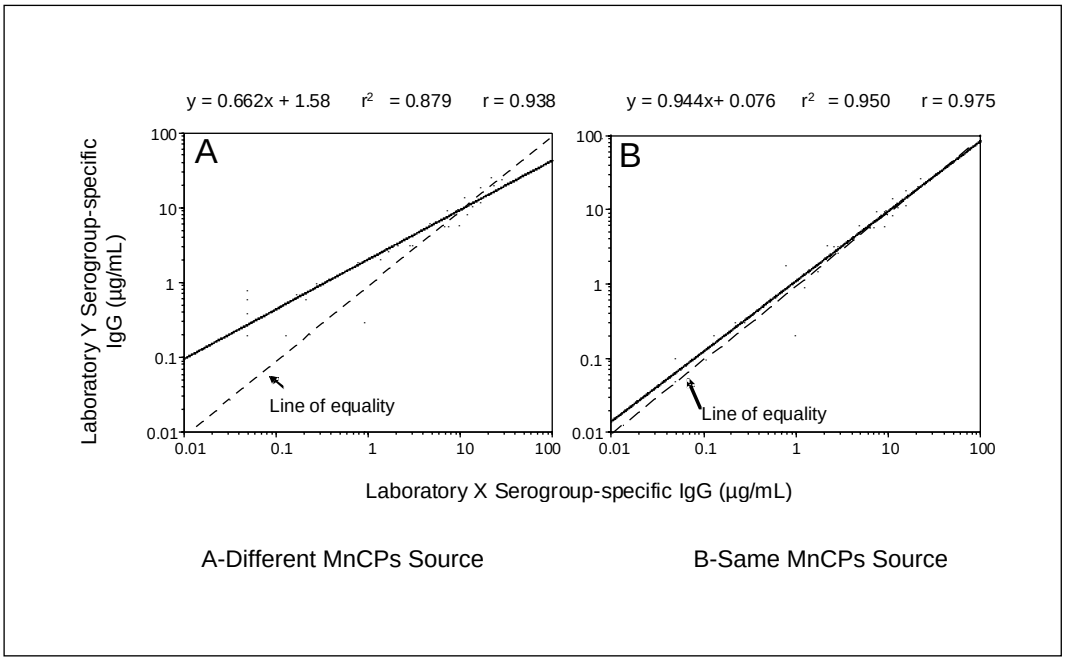


Post-immunization sera collected after a primary series or as part of the evaluation of a boosting dose show a similar relationship between the ELISA and SBA as observed in the adult immunized population. The correlation between ELISA and SBA is > 0.8.

For post-immunization sera our evaluations suggest that the ELISA could be used as a substitute for the SBA when the goal is to relate a significant ELISA-specific IgG concentration as a surrogate for SBA positive titre. We have analysed several sampling time points within infant MnCC studies (n = 450) to relate IgG concentrations ≥0.1 µg/ml to SBA titres of ≥1:4 or ≥1:8. Statistical analyses (cumulative distribution and regression) demonstrate that 1.2-2.0 µg/ml predicts an SBA ≥1:4 with a 95% confidence interval.

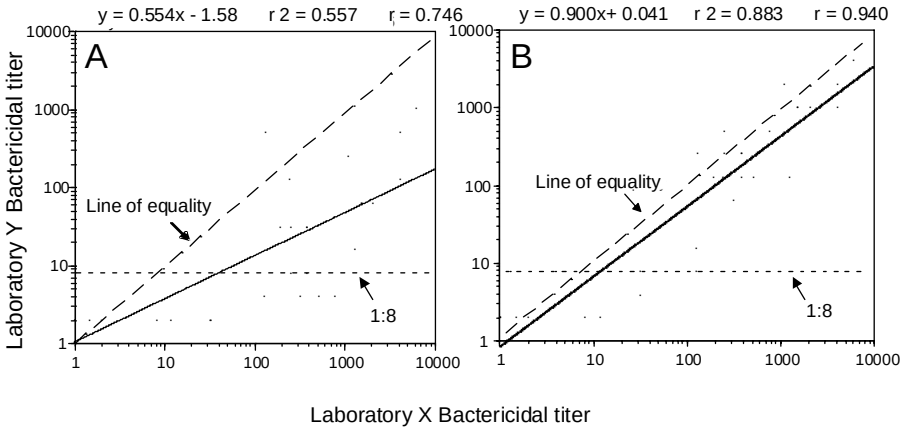
Certain parameters as well as procedural differences may have a large impact on the performance of assays. For ELISA, C polysaccharide integrity (purity, aseptic storage, coating in pyrogen-free water) and plate type are critical.

Figure 17: Impact of Quality of Antigen and Coating Conditions



For SBAs, critical parameters include the consistency of the strain (C11), the complement source and concentration, and the growth conditions (agar vs. broth).

Figure 18: Impact of Procedural Differences in SBA



A - C11 Strain grown in Broth (Laboratory Y) or on Agar (Laboratory X)

B - Agar grown C11 Strain used by both laboratories

For vaccine evaluations in infants and children, therefore, post-immunization sera should be evaluated in the context of serology measures from age-matched controls, and it is desirable to have subgroups assessed by the SBA. The quality of reagents and adherence to validated protocols are critical for optimal and consistent assay performance. However, the ELISA can serve as a surrogate for the more cumbersome and less quantitative SBA once a precise relationship has been defined. The precise relationship between the two assay methods should be reconfirmed by SBA testing of representative subsets of sera.

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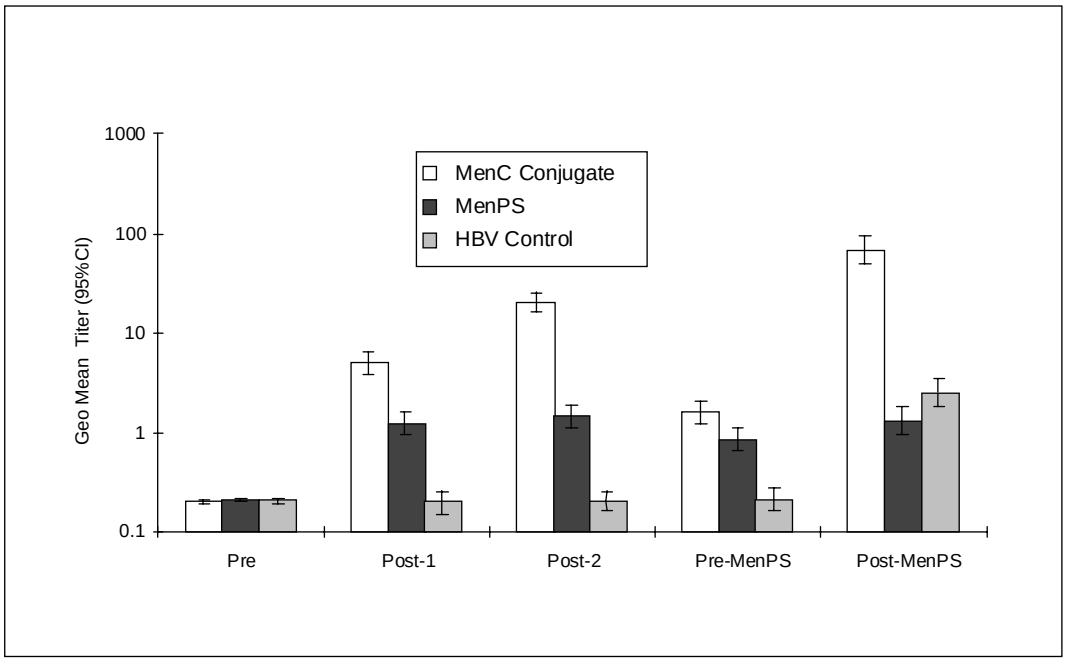
3.5.5 Chiron Vaccines Corporation[11]

Our laboratory has developed ELISA and bactericidal (BCA) assays to assess the serum antibody responses to a *Neisseria meningitidis* serogroup C (MenC) polysaccharide-CRM197 polysaccharide-protein conjugate vaccine in infants, children and adults. The ELISA assay is performed using MenC polysaccharide (Ps) that has been derivatized with adipic dihydrazide in the presence of 1-ethyl-3 (3-dimethyl-an-dnopropyl) carbodiimide. This modification allows the negatively charged MenC Ps to adhere to Dynatech Immulon II ELISA plates. Plates coated with 0.1 mcg/well of derivatized Ps are blocked with 1% bovine serum albumin (BSA) in 0.01 M phosphate-buffered saline (PBS). Serum samples are diluted in PBS with 1% BSA plus 0.1% Tween-20 and 75 mM NH_4SCN. The chaotrope inhibits the binding of low-avidity antibodies to the MenC Ps. The plates are developed with alkaline phosphatase-labelled mouse monoclonal anti-human IgG and p-nitrophenyl phosphate substrate. Sera are tested in twofold serial dilution on two separate plates and results are calculated on the basis of a standard curve on each plate. Each plate also includes high, low and negative controls and reagent blanks. The standard was prepared from pooled adult sera and was normalized against the CDC1992 reference serum (1) in the absence of NH_4SCN. The standard antiserum was assigned a value of 39 ELISA units/ml relative to CDC1992 in the absence of NH_4SCN. The value assigned to the standard antiserum was corrected to 30.8 ELISA units/ml in the presence of NF_4SCN (2). The concentration of chaotrope was selected to give the maximum differential ELISA activity between pools of toddler sera with BCA titres of < :8 and >= L8 respectively.

The addition of MenC Ps at a concentration of 25 mcg/ml to selected samples reduced the ELISA titre by 80-100%, indicating that the detected antibodies recognized unconjugated MenC Ps. Todders aged 15-23 months given two immunizations with MenC-CRM conjugate vaccine had a GMT of 20 U/ml one month after the second dose, while children of similar age given MenC Ps vaccine had a GMT of 1.5 U/ml (3).

11 John J. Donnelly, George Santos, William Wacknov, R. Randall Deck, Dan M. Granoff, Lisa Danzig, Andria Langenberg, Howard Raff.

Fig. 19 IgG Antibody Responses in Toddlers (15-23 months) Immunized with Two Doses of MenC Conjugate Vaccine



The BCA assay is performed using human complement as described by Goldschneider et al. (4) with modifications (5). MenC strain 60E bacteria are recovered from frozen stock overnight on chocolate agar in 5% $C0_2$ and inoculated into Mueller-Hinton broth with 0.25% D-glucose. Broth cultures are incubated at 37(C for 90 minutes in 5% $C0_2$ with agitation, centrifuged and resuspended in Gey's balanced salt solution (GBSS) with 1% BSA. Test sera (heat-inactivated at 56(C for 30 minutes) are serially twofold diluted in GBSS plus 1% BSA on microtitre plates. 25 mcl human complement (C) and 25 mcl bacterial suspension are added to 75 mcl of diluted sample. Each assay contains C' alone, heat-inactivated C, high, low and negative BCA titre, and buffer controls. Samples are tested in duplicate. Controls are sampled and plated on Mueller-Hinton agar at the start of incubation (0 time) and after 60 minutes of incubation at 37(C, and test wells are sampled and plated after 60 minutes of incubation. The percentage kill is calculated using the colony count at 60 minutes as the numerator and the average colony count at 0 time as the denominator. Titres are estimated as the serum dilution giving a 50% reduction in colony counts from the 0 time point. Acceptance criteria currently in use require that the time colony counts are between 30 and 100 colony forming units (CFUs), that the controls show at least a twofold increase in CFUs at 60 minutes, and that the values for negative and low-antibody and high-antibody controls fall within specified acceptance ranges.

A comparison of the Chiron modified BCA assay with that described by Goldschneider et al. is shown below.

Figure 20: Comparison of Results:
Chiron BCA vs. Goldschneider Method

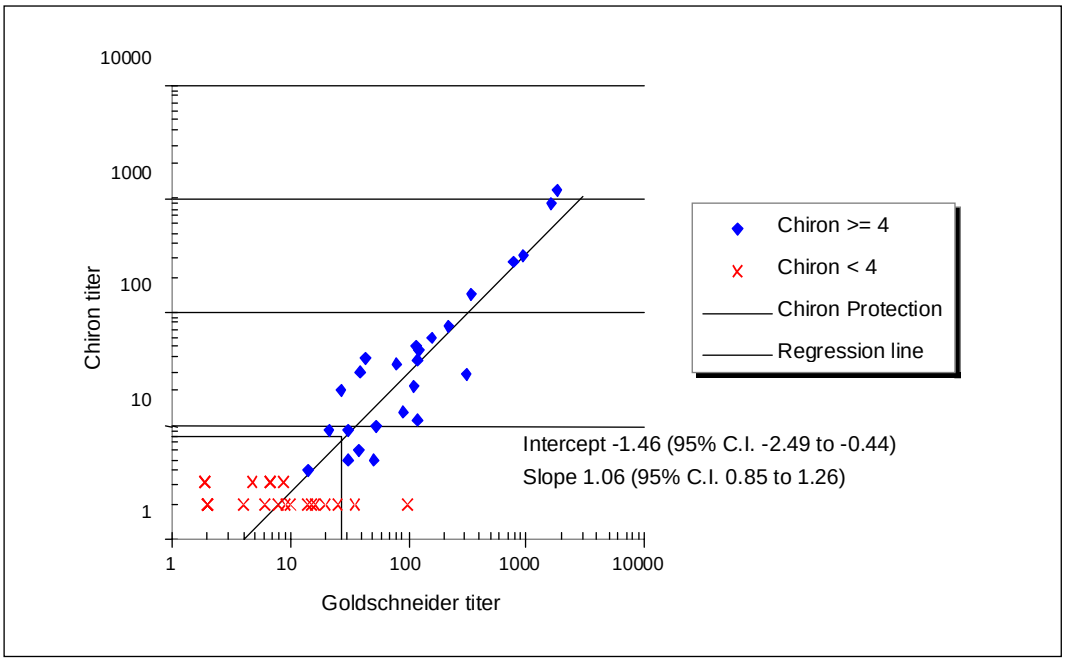
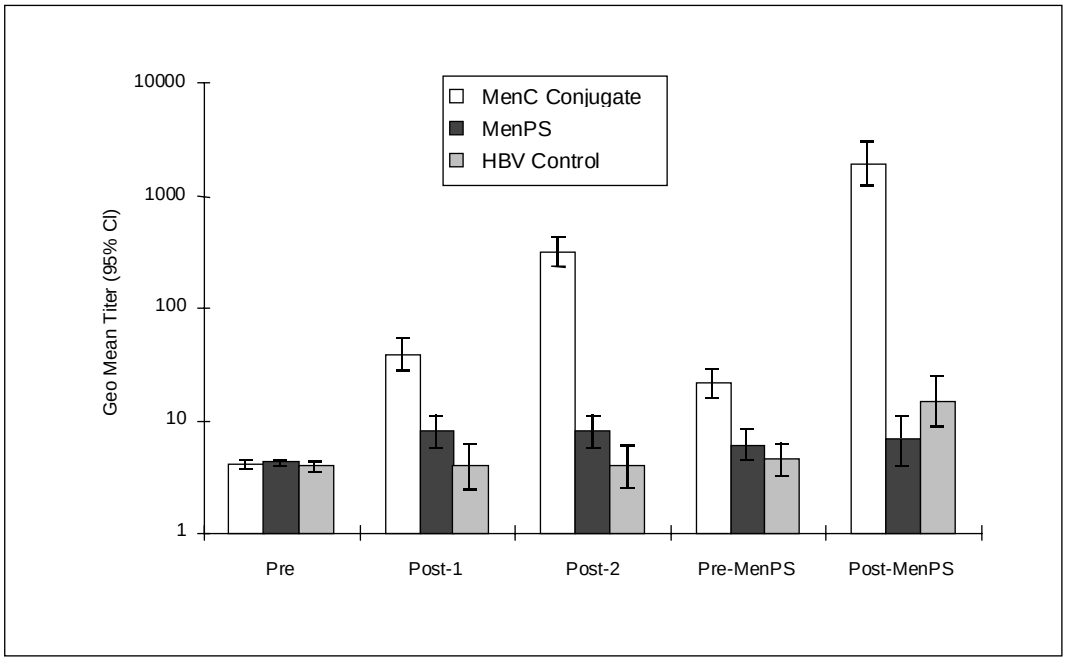


Table 9: Comparison of Chiron and Goldschneider BCA Methods in Vaccinees

Vaccine	Men C		Men PS	
Method	**Goldschneider**	**Chiron**	**Goldschneider**	**Chiron**
GMT	85	25	10	3
SD	5	6	4	2
N	26	26	20	20
P (1-tail paried t)	<<0.01		<<0.01	
Mean fold diff	3.4		3.8	
95% CI	2.7 - 4.4		3.0 - 4.9	

Toddlers aged 15-23 who received two immunizations with MenC-CRM conjugate vaccine demonstrated primary immunogenicity, persistence of antibody and immunological memory (3).

Figure 21: BCA Responses in Toddlers (15-23 months) Immunized with Two Doses of MenC Conjugate Vaccine



A comparison of ELISA and BCA results for toddlers given MenC conjugate vaccine and polysaccharide vaccine showed a correlation coefficient (Pearson r) of ca. 0.85. The BCA is relatively labour-intensive and is difficult to perform efficiently on large numbers of specimens. It may therefore be appropriate to use this ELISA, with its ability to detect high-avidity antibodies, to supplement the BCA as a measurement of antibody response in sera of vaccinees given MenC Ps protein conjugate vaccines.

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3.5.6 RIVM[12]

Neisseria meningitidis is a human pathogen and one of the major causes of bacterial meningitis. For meningitis A and C, polysaccharide conjugate vaccines have been developed with increased immunogenicity. The presence of serum bactericidal (SB) antibodies strongly correlates with clinical protection (1), and these protective antibodies are directed against the capsular polysaccharide. SB antibody titres of 1:4 or more are associated with protection (1,2). However, applicability to *Neisseria meningitidis* serogroup B is not established as the capsular polysaccharide is poorly immunogenic and protective antibodies must be directed at subcapsular antigens. Vaccines based on non-capsular surface antigens have been developed and used in several trials. The immunogenicity of two efficacious outer membrane protein-based serogroup B meningococcal vaccines (Finlay and Norwegian vaccine) were tested in Iceland and Chile and the SBA and ELISA antibody levels elicited were measured (3,4). Correlation between protection and SB antibody or ELISA titres depend on the vaccine used.

A hexavalent vesicle vaccine has been developed by RIVM in which six PorA-proteins (P1.7,16; P1.5,2; P1.19,15; P1.5 c,10; P1.12,13; P1.h7,4) are embedded in outer membrane vesicles (5,6). Recently, immunogenicity studies with different immunization schedules have been carried out in English infants (4 doses) and Dutch toddlers (3 doses; n = 189, 2-3 years) and schoolchildren (3 doses; n = 168, 7-8 years). Infants were vaccinated at age 2, 3, 4 and 15 months, the older children at 0, 2 and 8 months, with 50 or 100 µg total protein. To evaluate the immunogenicity of the vaccine, SBA titres against isogenic variants of strain H44/76, in which each vaccine PorA-protein is expressed individually, were measured at PHL (Manchester) and RIVM (Bilthoven).

The hexavalent vaccine was shown to be immunogenic, although multiple doses of vaccine were required to induce a significant SBA response and there were differences in the magnitudes of SBA responses to different PorAs (7, 8). The SBA were PorA-specific as no reaction was found with the PorA-deficient H44/76 variant HI5. The SBA titres obtained in the UK infants before and after vaccination were consistently higher (twofold to fourfold) than those obtained in Rotterdam. To eliminate he possibility that the difference in SBA titres was due to bactericidal assay, a comparison was made between the meningitis B bactericidal assay performed at PHL (Manchester) and that performed at RIVM (Bilthoven). The most important differences were the amount of human complement (25% versus 10 %) and the cut-off criterion (50% versus 90%). No significant differences in SBA were found between the sources of complement in the UK and the Netherlands. Using the Dutch protocol, 10 % of human complement was sufficient to kill the bacteria. However, the cut-off criterion is of major importance. Both the GMT and the percentage of children with a titre of 4 or higher were lower at the 90 % cut-off. In the Netherlands we found low pre-vaccination and post-vaccination titres; in the UK both were higher. This method gives comparable results even for 50% and 90% killing. Standardization and validation of the protocol used at RIVM will continue with sera obtained from CDC from the Chile and Iceland study where meningitis B vesicle vaccines from Norway and Cuba were used (3,4).

12 Prepared by the Laboratory for Vaccine Research and the Laboratory for Clinical Vaccine Research at the RIVM, Bilthoven, Netherlands, and the study teams of PHL Gloucestershire, and Sophia Children's Hospital, Rotterdam, Netherlands.

Total antibody responses were also measured by ELISA on trivalent OMV or whole cells. In the sera from the Rotterdam study we found no correlation between the bactericidal titres and the levels of total IgG antibodies measured by our ELISA. However, Aase et al. found a good correlation between SBA and anti-OMV IgG antibodies in adult volunteers immunized with the Norwegian vaccine (9). The amount of antibodies measured by ELISA is always higher than that indicated by the bactericidal assay, as not all IgG isotypes are equally effective in protective effector functions like complement-mediated bactericidal activity. We also investigated whether the variance in magnitude of bactericidal activity of the various PorAs could be caused by differences in isotype distribution. Using sera from the Rotterdam study we found that the highest bactericidal titres were found against P1.5c,10, followed by P1.12,13 and the lowest response was found to P1.h7,4 in children. The isotype distribution was similar for all three tested PorAs. IgG_1 antibodies dominated the response, followed by IgG_3 and low levels of IgG_2. No IgG_4 was detected. The isotype distribution induced by the RIVM hexavalent vesicle vaccine was comparable with that found after immunization with the Norwegian group B meningococcal outer membrane vesicle vaccine in volunteers (10,11,12). We could not find a correlation between isotype-specific antibody titres and SBA for the Rotterdam sera. A better correlation may be found when an epitope-specific ELISA is used..

Measurement of VR1 and VR2 specific antibody responses may also be of importance. For the bactericidal assay we have a set of isogenic PorA loop-deficient strains of H44/76 lacking the predicted loop 1 or loop 4 or both of PorA of serosubtypes P1.5c,10 and P1.7,16 (13,14). For P1.5 c,10 the bactericidal antibodies from vaccinees were mainly directed against loop 4. Previous studies have shown that for PorA P1.7,16 bactericidal antibodies were mainly induced by loop 1 (13,14). In both cases the bactericidal activity is highest against the longest (predicted) surface exposed loop of the protein. Point mutations and variants are known for several PorA types. The effect of these changes on the bactericidal activity of sera of vaccinees should be determined.

Through worldwide collaboration it should be possible to establish laboratory correlates of protection after vaccination with meningitis B vaccines. These correlates are needed for the evaluation of new meningococcal vaccines and childhood vaccine combinations. The final goal is to be able to predict protection via laboratory data in small-scale phase II trials so as to diminish the requirement for large-scale efficacy trials.

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3.6 Discussion

3.6.1 ELISA and SBA assays

The first meningococcal serogroup A and C polysaccharide vaccines were licensed on the basis of radioimmunoassay (RIA) and SBA. Only RIA and ELISA accurately measure capsule-specific antibody levels in human serum, while a bactericidal assay has historically been used to determine the functional antibody titres. Specific concerns about each of these assays need to be addressed before they are used in place of formal clinical efficacy studies. During the next few years, scientific inferences and public health policies relating to meningococcal conjugate vaccines will be based on immunogenicity data. It is highly likely that in the UK the licensing of serogroup C conjugate vaccines will be based on immunogenicity data rather than formal protective efficacy studies. It is, however, important that assay requirements be not so prescriptive as to hinder manufacturers' progress with the development and licensing of improved vaccines. For both functional and antibody assays, criteria have to be established for licensing based on immunological surrogates. Seroconversion rates and geometric mean titres elicited by new vaccines should be at least as high as for existing ones, while criteria for immunoglobulin isotype distribution, immunological priming and functional activity should be specified.

Complement-mediated bacterial killing has been demonstrated to correlate with protection against meningococcal disease. Therefore it seems reasonable to admit that an assay that determines serum bactericidal activity should constitute the "gold standard". However, many factors can affect the measurement of bactericidal activity. The choice of test strain, the growth conditions (e.g. the use of solid or liquid culture medium), the number of colony-forming units used, and the sources of exogenous complement were discussed in particular. In 1976, WHO's Expert Committee on Biological Standardization recommended a serum bactericidal assay for the production and release of batches of meningococcal polysaccharide vaccines, which has also been used to support licensing. For licensing, >90% of adult vaccinees should have a fourfold or greater rise in antibody titre. Unfortunately, researchers have continued to use numerous different bactericidal assay procedures without a formal comparison with the WHO method. The group felt that there was an urgent need to standardize this procedure between laboratories.

The relative advantages and disadvantages of using human or rabbit complement were debated and, if human complement were to be used, whether government agencies such as NIBSC or CDC should develop an international complement standard that individual laboratories could use to assess their local human complement sources. The principal concern was that the increased sensitivity of the assay using rabbit complement might lead to false positive results. However, since easily standardized batches of rabbit complement are commercially available (e.g. Pelfreeze), it was strongly suggested that a detailed statistical comparison be commissioned of data from assays using human and rabbit complement with a suitable range of serum samples. The purpose would be to investigate whether it was appropriate to establish a higher threshold titre when rabbit complement was used in the assay.

The question of whether the current serological assays adequately bridged to the original studies that established SBA as the surrogate of protection from infection was discussed. It was generally agreed that the Goldschneider study provided good evidence that the SBA correlates with protection but the standardized WHO and CDC assays differ in several critical details. The meeting agreed that the standard assay should be based on the CDC procedure but that it could be modified to accommodate the automation of plating and colony counting. Many of the participants considered that there were logistical difficulties with the use of the bactericidal assay to study very large numbers of serum samples, although this was not the unanimous view of the meeting.

It was agreed that a functional assay, such as the bactericidal procedure, would not be sufficient for the licensing of new conjugate vaccines. A reliable antibody assay provides valuable information on the quality of the antibody response and indicates whether the vaccine has stimulated immunological memory. The ELISA provides a sensitive, easily standardized and reproducible alternative which, if performed correctly, can provide a good surrogate of protection. In general, ELISA is more sensitive and less variable than the bactericidal assay; and is less labour-intensive and therefore more appropriate for the analysis of large numbers of serum samples from which a subset can be selected on a statistically appropriate basis for bactericidal assay. It can be used to provide information on isotype specificity as well as total serum antibody responses. In homogeneous populations of serum anticapsular antibodies, the ELISA correlates well with the SBA. However, in individuals with heterogeneous populations of capsule-specific antibodies, such as children immunized with plain polysaccharide vaccine, the correlation is poor. The most likely explanation is that administration of the plain polysaccharide vaccine to infants and young children elicits fundamentally low-avidity antibodies. Although these antibodies can be detected by ELISA they appear to be less active in functional bactericidal assays than anticapsular antibodies of higher avidity. Only an ELISA modified to measure only the high-avidity antibodies in serum can be used to predict a protective immune response. The following issues were raised.

1) There was agreement that the group would benefit from the support of a group of statisticians with experience of analysing data from these assays (see future follow-up).

2) Although the standard ELISA permits an evaluation of low-avidity antibodies in serum, this information is probably not important for licensing; the modified ELISA would be sufficient; two assays are not necessary.

3) The importance of relating antibody assays directly to bactericidal data and not to other antibody assays was stressed.

4) "High avidity" is a relative term and the correlation between a modified ELISA and SBA should be based on sound statistical advice.

5) Historically, the standard ELISA has been used to establish the mass of antigen-specific immunoglobulin in the response to a vaccine. It is not possible to use mass units with the modified ELISA and the principal concern with the use of this assay is to establish a consistent unitage.

6) Assays should not be vaccine-specific and should be able to accommodate future developments. They should also satisfy the guidelines of the International Conference on Harmonization of Technical Requirements for the Validation of Analytical Procedures (ICH)[13].

While there is every reason to be optimistic that the bactericidal and ELISA assays produce reliable data for serogroup C vaccines, considerably more research is needed before the assays can be implemented with confidence for serogroup A polysaccharide assays.

1) Current evidence suggests that the high-avidity ELISA as applied with C polysaccharide must be adapted if it is to provide reliable data on the immune response to serogroup A polysaccharide.

2) There should be a thorough review of the published evidence for correlates of protection for serogroup A meningococci, including epidemiological links.

3) More data are required on the immune response, including memory and antibody avidity.

4) Comparative data are required on the antibody responses to plain polysaccharide and conjugate serogroup A vaccines.

3.6.2 Reference reagents

Historically, the reference sera ECG and PB-2 have been used to estimate anticapsular antibody levels. These have been replaced by CDC1992, a serum pool from 14 adult volunteers who had received a single dose of tetravalent meningococcal polysaccharide vaccine, which has been compared with the earlier references to provide the necessary historical link. This reference is well characterized and available in sufficient quantity for use as a primary standard. The only possible drawback is that it was not raised against a conjugated vaccine but evidence suggests that it is suitable for use as a reference standard with either ELISA format and as a control in bactericidal assays. This reference has been available from CDC since 1992 and will continue to be available. Some of the plasma is currently being prepared for distribution from NIBSC as well.

13 The ICH was established in 1990 as a joint regulatory/industry project to improve, through harmonization, the efficiency of the process for developing and registering new medicinal products in Europe, Japan and the United States, in order to make these products available to patients with a minimum of delay. ICH topic Q2B 6, November 1996.

Twelve paired pre-immunization and post-immunization QC sera are available from CDC which were prepared in a similar manner to the reference. These sera reflect a range of titres but there was some debate about whether the range was adequate and whether it should be extended. It was argued that a pair of QC sera, one induced by the PS vaccine and one by the group C conjugate, were needed.

Serogroup A and C polysaccharide coating antigens, provided by Connaught Laboratories, are currently available from CDC. New batches of polysaccharides have recently been filled at NIBSC and will be compared with the CDC material. This should ensure a continuity of supply in the foreseeable future.

The question of using O-acetyl-negative group C meningococcal polysaccharides was also raised. Around 85% of serogroup C *N. meningitidis* strains produce an O-acetyl-positive polysaccharide. Although the use of deacetylated C polysaccharide was considered to be an interesting research question, for the purpose of standardizing the assays, O-acetyl-positive group C meningococcal polysaccharides should continue to be used.

A modified (high-avidity) assay was considered as the best ELISA technique for assessing functional antibody activity to group C polysaccharide. On the question of what antigen should be used in the solid phase, discussion focused on the use of methylated human serum albumin(mHSA)/polysaccharide or the derivatized antigen described in Granoff et al. (*Clin. diag. lab. immunol.*, 1998, 5 :479-485). The published method for the modified ELISA uses a derivatized meningoccal C polysaccharide as the solid phase antigen. Specificity of antibody binding is determined for each test serum by inhibition of binding by soluble native polysaccharide. The standard ELISA uses meningococcal C polysaccharide mixed with mHSA as the solid-phase assay, which is easier to prepare than a derivatized antigen. It was suggested that the mHSA/polysaccharide preparation could be used in a second-generation modified ELISA.

Whatever the antigen selected, it is essential that a reference laboratory provide sufficient mHSA or derivatized antigen to other laboratories for performing the assay locally, or assuring quality control of test antigen prepared locally.

Meningococcal strains for bactericidal assays are widely available. As to whether a reference serogroup C strain at a determined passage number should be made available to laboratories performing the SBA, the group felt that the number of passages has so far not proven a factor that would affect SBA titres. It was mentioned that C-11 (60E) has been widely used. Dr Carlone has this strain in lyophilized form and his laboratory could be used as a centralized location for storage and distribution.

4. Alternative assays

4.1 Infant rat meningitis passive protection assay[14]

Several group B meningococcal vaccine candidates based on outer membrane protein complexes have been evaluated in efficacy trials, and one is widely used in Latin America (1-4). The antibody response in connection with these trials has been extensively analysed but there is still much uncertainty concerning the requirements for protective immunity. The measurement of bactericidal or opsonophagocytic activity is difficult to standardize and their correlation to protection is not fully clear.

Animal models for meningococcal infection have been developed but are mostly considered unsatisfactory because of either artificial set-up (5,6) or the need for large inocula (7) or the use of adjuvants, e.g. mucin or iron (8,9). In our laboratory, Saukkonen et al. developed a meningitis model using rat pups aged four to six days (10). The pups are randomly redistributed in groups of six and injected i.p. with 100 µl of antibody preparation (usually polyclonal or monoclonal antibodies). One hour later a bacterial challenge is injected i.p. in a volume of 100 µl. The development of bacteraemia and meningitis is assessed by culturing samples of blood and cerebrospinal fluid taken six hours after challenge.

In this model, meningococci of groups A, B and C were found to be able to cause infection; the infectivity could in several cases be enhanced by passage of the bacteria in the rat pups. In the same model, Saukkonen et al. compared antibodies to capsular polysaccharides (PS), lipopolysaccharide (LPS) and outer membrane proteins (PorB and PorA) and found that protection was regularly seen with antibodies to PS and PorA (11,12). Subsequently this model has been used as a guide in the development work aiming at a PorA-based recombinant vaccine (13,14).

We have extended these studies to find out if the passive protection model could be used for human sera. We feel that a direct assay of the ability of these sera to protect in the infant rat model when administered i.p. has a good chance of providing a correlation of protection in humans. The optimization of the assay for human sera has been done already (15). With four well-defined sera from Norwegian vaccinees we have shown that challenge doses of 10^5 to 10^6/pup are optimal, that serum dilutions of 1/10 or 1/30 can be used, and that the reproducibility of the assay is satisfactory. We have also compared different strains of phenotypes B:15:P1.7,16:L3,7,9 and B:4:P1.15:L3,7,9 to optimize the assay for studying sera from vaccinees that have received Norwegian or Cuban group B meningococcal vaccine. By using the vaccine

14 Prepared by Helena Käyhty, Maija Toropainen and Helena Mäkelä.

strains we have now analysed paired sera from 97 subjects (i.e. a randomized 25% sample of all participants) from the Icelandic meningitis B vaccine study. The code has not been opened yet and thus we cannot give vaccine-specific results. However, the rate of positive blood cultures was higher in pre-immunization than in post-immunization sera and the colony-forming units per ml of blood were significantly lower in the post-immunization than in the pre-immunization sera (Table 10).

Table 10. The geometric mean (GM) level of bacteraemia in rat pups challenged with either strain H44/76-SL or Cu385 and passively immunized with pre- and post-vaccination (post- second) sera from the 97 vaccinees of the Icelandic study.

	GM (95%CI) of CFU x 105/ml of blood after challenge with:	
Serum	**H44/76-SL**	**Cu385**
Pre-immunization	1.02 (0.59-1.75)	1.13 (0.8-1.6)
Post-second dose	0.65 (0.36-1.16)	0.49 (0.31-0.75)

The model has also been used for studying the protection of anti-P1.16 antibodies against variant strains (16; P1.16a, b, c and d) carrying only one amino acid change in the surface loop of P1.16 PorA (17).

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4.2 Opsonophagocytosis of meningococci: correlation between flow cytometric opsonic activity and bactericidal activity[15]

Opsonization of microorganisms is considered one of the major protective functions of antibodies in the host defence system (1). The potential importance of complement-dependent opsonophagocytosis of meningococci in humans was recognized in the early 1900s (2,3). Resistance to disease and recovery from disease was found to be influenced, in part, by serum opsonins (3). Both human and animal studies were carried out using a variety of methods, techniques and complement sources.

Protection from meningococcal serogroup A and C disease is critically dependent on capsular polysaccharide antibodies. Immunization with meningococcal polysaccharide vaccines elicits complement-dependent serum bactericidal and opsonophagocytic antibodies. Both mechanisms operate simultaneously and function to clear meningococci. An intact complement pathway is required for optimal serum bactericidal activity, whereas only early complement components (deposition of C3) are required for opsonophagocytic activity. Sera from individuals with late complement-component deficiency have low or no serum bactericidal activity but do have opsonophagocytic activity. We developed and evaluated a complement-dependent, polysaccharide-specific flow cytometric opsonophagocytic assay that is not influenced by serum bactericidal activity by using serogroup A or C meningococcal polysaccharides covalently bound to fluorescent polystyrene beads.

The polysaccharide beads were phagocytized by activated HL-60 cells (promyelocytic leukaemia cells) chemically differentiated with sodium butyrate into monocytes. After inhibition with homologous polysaccharide, titres were reduced by >98% for serogroups A and C (n = 5). The opsonophagocytic assay, using baby rabbit complement, was shown to be highly reproducible after repetitive testing of a single quality control serum for serogroup A (GMT = 1,505; 95% confidence interval = 905-2520) and for serogroup C (GMT = 1024; 95% confidence interval = 562-1881). The variability from the median titre was ± 1 dilution for 78% of serogroup A assays (n = 9) and 100% of serogroup C assays (n = 6). Bactericidal titres as measured by a standardized assay involving the use of baby rabbit complement were correlated with titres obtained from the flow cytometric opsonophagocytic assay in paired sera from healthy adults (n = 35) vaccinated with the quadrivalent meningococcal polysaccharide vaccine (data for infants vaccinated with the protein conjugate vaccine are pending). Flow cytometric opsonophagocytic assay titres correlated well with bactericidal titres for serogroup A ($r = 0.75$, $p < 0.001$) and serogroup C ($r = 0.79$, $p < 0 .001$). Although these were different functional assays, the calculated titres were not significantly different for serogroup A ($p = 0.44$) and for serogroup C ($p = .19$). The results of a standardized IgG ELISA (µg/ml) correlated well with those of the flow opsonophagocytosis assay for serogroup A ($r = 0.83$, $p < 0.001$) and serogroup C ($r = 0.74$, $p < 0.001$). The serum bactericidal assay also correlated well with the ELISA for serogroup A ($r = 0.89$, $p < 0.001$) and for serogroup C ($r = 0.85$, $p < 0.001$).

15 Prepared by George M. Carlone.

Baby rabbit and human complement sources were compared in the flow opsonophagocytic assay using 10 of the 35 sera previously assayed. The baby rabbit and human serum complement sources were initially screened by SBA to assure that they did not have any serum bactericidal activity without addition of serogroup-specific antibodies. Serum bactericidal titres correlated poorly when baby rabbit serum and human serum were used as complement sources in the SBA for serogroup A ($r = 0.15$, $p > 0.01$) and serogroup C ($r = 0.45$, $p > 0.01$). However, flow cytometric opsonophagocytic titres correlated well for serogroup A (data pending) and for serogroup C ($r = 0.84$, $p < 0.001$) when the two different complement sources were used. Similar opsonophagocytic titres were observed using either baby rabbit serum or human serum as complement, whereas SBA and opsonophagocytic titres differed (lower titres with human complement) when the two complement sources were used. It appears that the two complement sources differ in their activity above the level of C3.

The flow cytometric opsonophagocytic assay has a number of advantages over the SBA: 1) it requires less time (6 hours) to perform than the SBA (48 hours); 2) it uses non-infectious targets; 3) it is not affected by serum antibiotics; 4) it is semi-automated; 5) it can evaluate the production of functional antibodies for new meningococcal vaccines in complement-sufficient and late complement component-deficient individuals.

References

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4.3 Discussion

A number of animal models of meningococcal infection have been developed but most are considered unsatisfactory because they do not reflect natural infection requiring very large inocula or the use of excipients such as mucin or iron. A model was described in which rat pups aged 4-6 days were used in passive immunization studies. Serogroup A, B and C meningococci were able to cause infection in this model and protection could be seen when capsular polysaccharide-specific and PorA-specific antibodies were administered i.p. The infant rat passive protection assay had considerable merit as a research procedure but had not been sufficiently evaluated with human sera and was impractical for handling the large numbers of serum samples from clinical trials needed to support licensing of A and C conjugate vaccines.

Serum bactericidal and opsonophagocytic antibodies specific for capsular polysaccharides function together to clear meningococci and thereby protect the host from infection. An intact complement pathway is required for efficient bactericidal killing, while only the early components are required for opsonophagocytic activity. A complement-dependent flow cytometric assay was described using fluorescent polystyrene beads coated with either serogroup A or serogroup C polysaccharides. It provides an alternative functional assay to the bactericidal assay which is independent of SBA and is not affected by the presence of antibiotics. Unlike the bactericidal assay, it is not sensitive to the source of complement, suggesting that the differences observed between bactericidal assays using different complement sources arise after the deposition of the C3 component. It has a number of other advantages: it is safe as it does not involve the use of infectious agents; it is performed more quickly than the bactericidal assay; and it can be semi-automated. The question was raised as to how widely available flow cytometry would be for laboratories testing sera from clinical trials. It was considered that, given the wide acceptance of the bactericidal assay, an opsonophagocytic assay, while a useful adjunct, would not replace it as the approach of choice. It may, however, play an important role in the development of a functional assay for serogroup A antibodies.

5. Conclusions and recommendations

1) Efficacy studies are not needed for licensing serogroup A and C meningococcal conjugate vaccines because of compelling data indicating that serum anticapsular antibodies confer protection.

2) The principal but not the sole function of antibody assays is to support licensing.

3) Both serum bactericidal assay (SBA) and ELISA data will be needed for licensing.

4) The conclusions listed below are intended to be applicable to foreseeable combination vaccines and novel formulations.

5) The SBA is the functional assay of choice at present and results are considered to predict protection.

6) Developmental work should continue on other functional assays.

7) The adoption of a standard SBA technique was agreed.

8) The CDC (1997) group C SBA assay with a provision for a modification to allow semi-automation of colony counting was adopted as the optimal methodology.

9) Baby rabbit serum (BRS) will be the source of exogenous complement.

10) To avoid overestimating protection with BRS complement, a threshold titre correlating with protection will be determined by reference to SBA data obtained with human complement.

11) An SBA study design for collaborative evaluation of rabbit and human complement will be developed.

12) ELISA results should predict functional antibody activity.

13) A modified (high-avidity) assay was adopted as the best ELISA technique for assessing functional antibody activity to group C polysaccharide.

14) Reference laboratories should standardize on a single high-avidity ELISA protocol for group C polysaccharide.

15) The standard ELISA offers important additional information on human responses to meningococcal A/C polysaccharide and conjugate vaccines.

16) Reference polysaccharides are available and should be used for standardization.

17) The CDC 1992 reference serum was regarded as the most important ELISA external quality standard.

18) Data derived from modified ELISAs should be expressed in units that are clearly distinct from, but mapped to, mass units by means of the CDC1992 reference serum.

19) Laboratories are encouraged to use the CDC1992 serum pool as their primary reference serum.

20) For both the ELISA and the SBA, additional quality control sera with lower antibody levels are required, as are sera containing low-avidity antibodies (one interlaboratory comparison will be required).

21) Adherence to ICH guidelines on the validation of assay methods is recommended.

22) Modifications to current group A SBAs and ELISAs need to be evaluated.

23) A source of human complement for group A SBA is needed.

24) More data are needed on group A: population immunity, priming and memory to polysaccharide and antibody avidity.

25) Additional epidemiological studies are needed to link clinical efficacy with serological correlates of protection against group A and group C disease.

6. Future follow-up

A group of statisticians is being formed by CDC, WHO and FDA, coordinated by Dr B. Plykatis. Statisticians from other groups are welcomed to join this initiative. Its mandate comprises, in principle, giving support to the following activities:

- determination of a threshold value for the comparison of rabbit and human complement (analyses are currently being performed by Dr Plykatis in collaboration with Drs Granoff, Carlone and Borrow; As soon as definite conclusions are possible the results will be made available to the group);
- development and validation of an analytical model to determine whether different laboratories can generate data in agreement with one another;
- keeping good statistical control over all proposed research projects;
- design and analysis of multilaboratory studies for validation of immunoassays for future vaccine evaluation and licensing.

An SBA study design for collaborative evaluation of rabbit and human complement as well as additional quality control sera with lower antibody levels for both ELISA and SBA are being undertaken by Drs Granoff, Carlone and Borrow.

With regard to the high-avidity ELISA protocol for serogroup C, a study comparing the performance in predicting bactericidal titres of a modified ELISA using mHSA/polysaccharide or derivatized polysaccharide will be undertaken.

(The protocol for preparing basic esterified methylated human serum albumin is given in Annex 3. The three changes proposed by the Manchester Public Health Laboratory to a modified ELISA using mHSA/polysaccharide are: 1) use of Tween 20 instead of Brij 35; 2) addition of 75 mM ammonium thiocyanate to the serum diluent buffer; 3) change of CDC 1992 from serogroup C specific IgG value of 24.1 µg/ml to 19.6 EU/ml. The SOP for these modifications is attached as Annex 4.)

Current serogroup A SBA and ELISA protocols will be carefully revised. Several laboratories (Children's Hospital Oakland Research Institute, NIBSC, CDC, Manchester Public Health Laboratory, and possibly others) have offered to assay a panel of selected sera to confirm that the observations presented at the meeting can be reproduced. The panel of sera will represent different age groups (infants, toddlers, adults), avidities and vaccines (polysaccharide and conjugate). The SBA assay will be evaluated using both human and rabbit complement sources and the threshold value for protection will be determined as for serogroup C (group of statisticians).

At least one laboratory should evaluate several target strains. These strains will be well characterized epidemiologically (strains from African outbreak) and may not be limited to laboratory strains. In the SBA study by the multilaboratory study group (Maslanka et al. *Clin. diag. lab. immunol.*, 1997, 4: 156-167) the vaccine type-strain A1 was evaluated using rabbit serum as a source of complement (extremely high titres were observed, even with pre-vaccination sera). SBA values, using human complement, need to be determined.

Research on additional assays (animal models and opsonophagocytic assay) will continue by the National Public Health Institute in Finland and the CDC.

Despite the interest of all the above institutions in collaborating to solve these problems, additional resources will have to be mobilized for the successful performance of all research studies. During the meeting it was agreed that WHO should try to make funds available for both setting up a limited network of statisticians and stimulating research on these topics. Proposals on all the above unsolved issues will be submitted to WHO from interested parties, detailing procedures, partners and budget requirements. These proposals will be compiled in a single package and sent to all potential donors, including the International Federation of Pharmaceutical Manufacturers Association (IFPMA).

Annex 1:
Agenda

Monday 8 March 1999

8.30-8..50	Opening of the meeting • Presentation of participants • Adoption of the agenda	Dr L. Jódar Dr K. Cartwright
8.50-9..35	Pathogenesis of meningococcal infection and mechanisms of host immunity	Dr P. Densen
9.35-10.00	Recent trends in meningococcal disease epidemiology and implications for use of meningococcal vaccines	Dr J. Wenger
10.00-10.30	*Coffee break*	
10.30-11.15	Current assays revisited: scientific concerns and practical issues	Dr D. Granoff
11.15-12.00	ELISA and bactericidal assays: how did we get where we are?	Dr G. Carlone
12.00-12.45	Evaluation of human antibody response to serogroup C *Neisseria meningitidis* invasive infection using different immunological assays	Dr C. Frasch
12.45-13.30	Meningococcal serogroup A/C vaccine assay status - the UK experience	Dr R. Borrow
13.30-14.30	*Lunch*	
14.30-15:15	Other functional assays: • Infant rat meningitis passive protection assay • Opsonophagocytosis of meningococci: correlation between flow cytometric opsonic activity and bactericidal activity Industry presentations on serological assays data	 Dr H. Käyhty Dr G. Carlone
15.15-15.35	Pasteur-Mériéux Connaught	Dr R. Ryall
15.35-15.50	*Coffee break*	

Monday 8 March 1999 (*continued*)

15.50-16.10	SmithKline Beecham	Dr M. Laferriere
16.10-16.30	NAVA	Dr P. Fusco
16.30-16.50	Wyeth Lederle	Dr D. Madore
16.50-17.10	Chiron Vaccines	Dr J. Donnelly
17.10-17.30	RIVM	Dr L. van Alphen
17.30-18.30	First general round table discussion: • Comments on presentations made on first day • Outstanding questions (Working group to draft conclusions of first day)	

Tuesday 9 March 1998

9.30-11.00	• Round table discussion on ELISA, SBAs and other assays • Research agenda for current and/or new assays	
11.00-11.15	*Coffee break*	
11.15-13.00	• Round table discussion (continuation) • Preliminary recommendations on current assays and research agenda	
13.00-15.00	*Lunch (working group to draft final recommendations)*	
15.00-17.00	Working group presentation of final recommendations and research agenda	Dr. L. Jódar Dr K. Cartwright
17.00	Closure of meeting	

Annex 2:
List of participants

Dr P Anderson, 100 Bayview Drive, PH 24, North Miami Beach, FL33160, USA
Tel. 1 305 944 7838; Fax: 1 305 944 7838; e-mail: 11074.1154@compuserve.com

Dr R. Borrow, Molecular Biology, Manchester Public Health Laboratory, Withington Hospital, Nell Lane, West Didsbury, GB-Manchester M20 2LR , UK
Tel. 44 161 291 4633; Fax +44 161 446 2180; e-mail: ray@manphl.demon.co.uk

Dr G Carlone, Chief, Respiratory Diseases Immunology Section, Respiratory Diseases Branch, Division of Bacterial and Mycotic Diseases, National Center for Disease Control and Prevention, Building 1, Room 1260, A36, Clifton Road, Atlanta, GA 30333, USA
Tel. 1 404 639 3622; Fax. 1 404 639 3115; e-mail: gmc3@cdc.gov

Dr K Cartwright, Public Health Laboratory, Gloucestershire Royal Hospital, Great Western Road, GB-Gloucester GL1 3NN, UK
Tel. 44 1452 305 334; Fax. 44 1452 307 213; e-mail: cdiett@phls.nhs.uk

Dr P. Densen, Associate Dean, College of Medicine, University of Iowa, CMAB 116, Iowa City, IA 52246-1101, USA
Tel. 1 319 335 6905; Fax. 1 319 335 8049; e-mail: peter-densen@uiowa.edu

Dr I Feavers, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, GB-Hertfordshire EN6 3QG
Tel. 44 1707 654 753; Fax. 44 1707 646 730; e-mail: ifeavers@nibsc.ac.uk

Dr C Frasch, Chief, Laboratory for Bacterial Polysaccharides, Center for Biologics Evaluation and Research, US Food and Drug Administration, 1401 Rockville Pike, HFM 428, Rockville, MD 20852, USA
Tel. 1 301 496 1920; Fax. 1 301 402 2776; e-mail: frasch@cber.fda.gov

Dr D Granoff, Children's Hospital Oakland Research Institute, 747 Fifty-second Street Oakland California 94609 USA.
Tel: (510) 428-3728; Fax: (510) 428-3608; e-mail: dgranoff@mail.cho.org

Dr J Holst, Head of Control, Department of Vaccinology, National Institute of Public Health (NIPH), P.O. Box 4404, Torshov, N-0403 Oslo, Norway
Tel. 47 22 04 2423; Fax: 47 22 04 23 01; e-mail: jholst@online.no

Dr H. Käyhty, National Public Health Institute, Mannerheimintie 166, FIN-00300 Helsinki, Finland
Tel. 358 9 4744 8237; Fax. 358 9 4744 8599; e-mail: helena.kayhty@ktl.fi

Dr B Perkins, Chief, Meningitis and Special Pathogens Branch, Division of Bacterial and Mycotic Diseases, Center for Disease Control and Prevention, Atlanta, GA 30333, USA
Tel. 1 404 639 4721; Fax. 1 404 639 0817; e-mail: bap4@cdc.gov

Observers

Dr A. Bartoloni, Process Development Department, Chiron Vaccines, Via Fiorentina 1, I-53100 Siena, Italy
Tel. 39 577 243 380; Fax. 39 577 243 317; e-mail: bartolon@iris02.Biocine.it

Dr M Bybel, Head of Serology, Pasteur Mérieux Connaught-USA, Route 611, P.O. Box 187, Swiftwater, PA 18370, USA
Tel. 1 717 839 5015; Fax. 1 717 839 0619; email: mbybel@usa.pmc-vacinnes.com

Dr P. Costantino, Process Development Department, Chiron Vaccines, Via Fiorentina 1, I-53100, Italy
Tel. 39 577 243 380; Fax. 39 577 243 317;
e-mail: Paolo_Costantino@iris02.Biocine.it

Dr John J. Donnelly, Director, Vaccines Research, Chiron Corporation, 4560 Horton St., Emeryville CA 94608, USA
Tel: +510-923-8371; Fax: +510-658-0329; email: john_donnelly@cc.chiron.com

Dr C. Ethevenaux, Pasteur Mérieux Connaught France, 1541 avenue Marcel Mérieux, 69280 Marcy l'Etoile, France
Tel: + 33 4 37 37 70 64; Fax: + 33 4 37 37 78 88;
e-mail: cethevenaux@fr.pmc-vacc.com

Dr M. Hohenboken, Director, Clinical Development, Pediatric Vaccines Devel. Unit, SmithKline Beecham Biologicals, R&D Immunology-Biology, Rue de l'Institut 89, B-1330 Rixensart, Belgium
Tel. 32 2 656 9138; Fax. 32 2 656 8133; e-mail: matthew.hohenboken@sbbio.be

Dr B. Fritzell, Chiron Vaccines, via Fiorentina, 1, I-53100 Siena, Italy
Tel. 39 577 243 222; Fax: 39 577 243 551

Dr P. Fusco, Director of Microbiology and Immunology, North American Vaccines, Inc., 12103 Indian Creek Court, Beltsville, MD, 20705, USA
Tel. 1 301 419 8400; Fax. 1 301 419 5040; e-mail: pfusco@nava.com

Dr M. Granstrom, Department of Clinical Microbiology, Karolinska Hospital, S-171 76 Stockholm, Sweden
Tel. 46 8 517 735 64; Fax. 46 8 30 80 99; e-mail: marta@mb.ks.se

Dr Laferriere, Preclinical Immunology and Bacteriology, SmithKline Beecham Biologicals, Rue de l'Institut 89, B-1330 Rixensart, Belgium
Tel. 32 2 656 9776; Fax. 32 2 656 9131; e-mail: laferrie@sbbio.be

Dr D. Madore, Wyeth Lederle, Director, Research Operations, Wyeth-Lederle Vaccines and Pediatrics, 211 Bailey Road, West Henrietta, NY 14586-9728, USA
Tel. 1 716 272 7000; Fax: 1 716 273 7515;
email: dace_madore@internetmail.pr.cyanamid.com

Dr K. Mayer, Chiron Behring GmbH & Co., Pre-Clinical Development, P.O. Box 1630, D-35006 Marburg, Germany
Tel. 49 64 21 39 34 39; Fax. 49 64 21 39 51 59

Dr G. Metz, SmithKline Beecham Biologicals, R&D Immunology-Biology, Rue de l'Institut 89, B-1330 Rixensart, Belgium
Tel. 32 2 656 9144; Fax. 32 2 656 7190; e-mail: metz@sbbio.be

Dr A. Michaelides, Biologist, Vaccines Division, Bureau of Biologics and Radiopharmaceuticals, Therapeutic Products Programme, LCDC, Bldg 6 (Locator 0603C2), Tunney's Pasture, Ottawa K1A OL2, Canada
Tel. 1 613 946 0481; Fax. 1 613 941 5841; e-mail: Alecos_Michaelides@hc-sc.gc.ca

Dr Moon H. Nahm, School of Medicine (Room 4-7219B), University of Rochester, 601 Elmwood Ave., Rochester NY 14642-8777, USA
Tel. 1 716-275-0414; Fax: 716-271-7512; email: moon@vaccine.rochester.edu

Dr Robert P. Ryall, Senior Research Scientist, Pasteur Mérieux Connaught-USA, Route 611, P.O. Box 187, Swiftwater, PA 18370, USA
Tel. 1 717 839 4566; Fax. 1 717 839 0619; email: rryall@usa.pmc-vacinnes.com

Dr L. van Alphen, RIVM, Antonie van Leeuwenhoeklaan 9, NL-3720 Bilthoven, The Netherlands
Tel. 31 30 274; Fax. 31 30 274 2971; email: loek.van.Alphen@rivm.nl

Secretariat

Dr E. Griffiths, Biological Unit, World Health Organization, 1211 Geneva 27, Switzerland
Tel. 41 22 791 3890; Fax. 41 22 791 0746; e-mail: griffithse@who.ch

Dr L. Jódar, Vaccine Development, Vaccines and Other Biologicals, World Health Organization, 1211 Geneva 27, Switzerland
Tel. 41 22 791 3744; Fax. 41 22 791 4860; e-mail: jodarl@who.ch

Dr K Mulholland, Vaccine Development, Vaccines and Other Biologicals, World Health Organization, 1211 Geneva 27, Switzerland
Tel. 41 22 791 4760; Fax. 41 22 791 4860; e-mail: mullhollandk@who.ch

Dr E. Tikhomirov, Bacterial Diseases Unit, World Health Organization, 1211 Geneva 27, Switzerland
Tel: 41 22 791 2688; Fax: 41 22 791 0746; email: tikhomirove@who.ch

Dr J. Wenger, Expanded Programme on Immunization, Global Programme for Vaccines and Immunization, 1211 Geneva 27, Switzerland
Tel. 41 22 791 4511; Fax. 41 22 791 4193; email: wengerj@who.ch

Annex 3:
Protocol for the preparation of basic esterified methylated human serum albumin

1. Suspend 5 g (0.25 g albumin/µL) human albumin in 500 ml absolute methyl alcohol and add 4.2 ml 12 N hydrochloric acid.

The protein dissolves and eventually precipitates again.

2. Allow the mixture to stand in the dark for 3 days or more with occasional shaking (once or twice a day).

3. Collect the precipitate in 250-ml centrifuge bottles, and wash twice with methyl alcohol and twice with anhydrous ether in the centrifuge bottles.

4. In a well-ventilated chemical hood, evaporate most of the ether in air or flush with dry nitrogen, and then *in vacuo* over KOH.

5. Reduce the material to a powder and store over KOH or lyophilize.

NOTE: Failure to remove the residual acid reduces the basicity of the final product.

Albumin, human (25% solution containing 0.25 g albumin/ml).
Central Laboratory, Blood Transfusion Service, Swiss Red Cross, Wankdorfstrasse 10, CH-3000, Berne 22.
Distributed by American Red Cross, Blood Services, Washington DC.

References

1. Mandell JD, Hershey AD. A fractionating column for analysis of nucleic acids. Anal. biochem., 1960, 1: 66-77.

2. Gheeling LL et al. Multicentre comparison of *Neisseria meningitidis* serogroup C anticapsular polysaccharide antibody levels measured by a standardized enzyme-linked immunosorbent assay. *J. clin. microbiol.*, 1994, 32: 1475-1482.

Annex 4:
Standard operating procedure, Manchester PHL

Modified CDC ELISA for detection of serogroup C-specific Ig
Department : Molecular Biology
Reference number: 489

1. Safety

- Safety working and the prevention of infection in clinical laboratories, HMSO 1991.
- Advisory Committee on Dangerous Pathogens, HMSO 1990.
- Public Health Laboratory Service Safety Precautions 1993.
- Manchester Public Health Laboratory Safety Policy SOP December 1997
- Manufacturer's data sheets.

Refer to COSHH assessment form numbers:

- 400: Precautions for handling blood and serum
- 252: Enzyme-linked immunosorbent assays

2. Aim

To measure the level of specific primarily high avidity IgG antibodies human sera (determined relative to a human reference serum pool) to *Neisseria meningitidis* serogroup C polysaccharide.

3. Specimen Requirements

Serum: The volume of serum needed for determination of IgG antibody against *Neisseria meningitidis* serogroup C Polysaccharide is 20 µL, optimal volume 50 µL. Upon receipt of a serum specimen in the Meningococcal Reference Unit (MRU), the serum will frozen at -80°C in a locked, alarmed freezer in room 257.

Serum specimens shipped to MRU from outside the Manchester PHL must be transported in compliance with the guidelines outlined in "Safety Precautions, Notes for, Guidance Public Health Laboratory Service (sections 80-92)", published and distributed by the PHLS. Upon receipt of a specimen in the MRU, the person taking receipt of the specimen will record the sample I.D. information and the date the specimen was received. The information available on the specimen request form will be entered in the Meningococcal Vaccine Database.

On the day of testing, the specimen will be thawed at room temperature, assayed and then immediately refrozen. No specimen should be frozen and thawed more than four times. The number of freeze/thaw cycles a specimen undergoes should be recorded on its container lid by a marker pen dot.

Upon completion of specimen testing the test result will be entered in the meningococcal serology database.

4. Materials

- Immulon 2 flat bottom 96-well polystyrene microtitration plates, Cat no. M129B, Dynex Technologies Ltd.
- Pressure sensitive film, Titertek plate sealers, Cat no 77-400-05 or equivalent, ICN
- SkanWasher 300 version B, Skatron
- 12-channel Titertek plus pipettes, ICN, 50 - 200 µL
- Single channel pipettes, 20, 100, 200 & 1000 µL, Gilson, Anachem
- Titertek multiskan ELISA reader - model MCC (type 340)
- Pipette tips, Cat no 08319 or equivalent, Sterilin
- Filter units (0.22 mm), Cat no GVWP04700 or equivalent, Millipore.
- ELISA for windows version 1.07. Data analysis software (provided by CDC, Atlanta, USA) - 4-parameter logistic curve model (Reference: *J. Clin. Microbiol.* 1991. 29 : 1439-1446).

5. Reagents

- Methylated Human Serum Albumin (mHSA):
  - Rehydrate vial with sterile, pyrogen-free water, to yield a stock solution of 10 mg/ml
  - Store at +4°C. Shelf life 3 months. (References: Analytical Biochemistry (1960) vol. 1, p. 66 and Journal of Clinical Microbiology (1992) vol. 30:1, p. 154.)
- Mab - Human IgG Fc PAN (1,2,3,4) HRP conjugated, Stratech Scientific Ltd, Cat no 6043HRP
- *Neisseria meningitidis* serogroup C polysaccharide. Store in aliquots at -80°C. Avoid excessive freeze/thawing.
- Tween 20 detergent (Polyoxyethylene-sorbitan monolaurate), Sigma, Cat no P-1379 or equivalent
- Phosphate buffered saline (PBS), Oxoid, Code BR 14a or equivalent.
  - (Dissolve 1 tablet in 100 ml of ddH_2O).
- Wash buffer (PBS, pH 7.4): containing 0.1 % Brij-35
- Pyrogen free water, Water for Injections BP, Phoenix Pharmaceuticals Ltd.
- Coating buffer, PBS (pH 7.4) made in pyrogen free water.
- Newborn bovine serum (NBBS), ICN, Cat no 29-121-54 or equivalent

- Ammonium thiocyanate, Sigma, Cat no A-0302.
- Serum buffer: 5 % NBBS, 0.05 % Tween 20, 75 mM Ammonium thiocyanate in PBS
- Conjugate buffer: 5 % NBBS, 0.05 % Tween 20 in PBS
- Substrate buffer (for 10 ml):
  - ddH_20 ..... 5 µL
  - 0.2 M Dibasic sodium phosphate (Sigma, Cat no S0876 or equivalent) ..... 2.57 µL
  - 0.1 M Citric acid (Sigma, Cat no C7129 or equivalent) ..... 2.43 µL
  - TMB tablet (1 mg) (Sigma, Cat no T3405 or equivalent) ..... 1 tablet
  - H_2O_2 ..... 2 µL
- 2 *M* sulphuric acid, BDH, Cat no 19815 4Y or equivalent.
- Standard Reference Serum (*Neisseria meningitidis*) Human Serum Pool CDC 1992:
  - Pool of donor sera. (Total serogroup C anti-capsular IgG antibody concentration = 19.6 EU/µL.)
  - Rehydrate vial with 1 µL of sterile double distilled water and aliquots. Store at -80°C in aliquots.

6. Sample Preparation

All reagents stored under refrigeration must be brought to room temperature before use.

Dilute Standard Reference Serum (*Neisseria meningitidis*) Human Serum Pool CDC1992 and serum samples in serum buffer. Prepare 2 x stocks of the CDC 1992 and serum samples as they will be diluted 1:1 with buffer in the first well of each dilution series.

NOTE: Make a 1:75 2 x pre-dilution of CDC 1992 serogroup C antibody detection. Make a 1:25 2 x pre-dilution of unknown serum samples for serogroup C.

7. Quality Control

Serum OM/3335 (A post vaccination "immune serum") is used as a quality control (QC) serum for the ELISA. The QC serum will be diluted with serum buffer in such a way as to yield optical densities in the high, middle, and low portions of the assay standard reference working curve (1/200, 1/1000, 1/4000).

Stock 1 x working solutions of QC serum are prepared, aliquoted, and frozen at -80°C. On the day of testing, remove, thaw an appropriate number of high, middle, and low QC level frozen aliquots. A high, middle, and low QC level is run in duplicate on every plate. Each plate will require 200 µL of each QC level run.

The antibody level for each QC level must not deviate by more than ±2 SD of its established mean.

The antibody level for a QC level may on occasion fall outside ±2 but within ±3 SD of its mean. Such an occurrence can usually be traced to a change in assay, i.e. new lot of plates, substrate tablets. Make note on worksheets of ALL assay lot number changes.

8. Assay procedure

8.1 Preparation of antigen-coated microtitre plates:

1) Coat Immulon 2 plates with a mixture of methylated Human Serum Albumin (mHSA) at a final concentration of 5 mg/µL and meningococcal polysaccharide serogroup C (10 m*M* PBS, pH 7.4) at a final concentration of 5 mg/µL in coating buffer [made from pyrogen free water]). The procedure for coating four plates is as follows:

   (a) Prepare a 10 mg/µL working solution of serogroup C polysaccharide by diluting 8 µL of the polysaccharide stock (25.0 mg/µL) in 20 µL of PBS, pH 7.4. Prepare a 10 µg/mL working solution of mHSA by diluting 20 µL of the mHSA stock (10.0 mg/mL) in 20 mL of PBS, pH 7.2-7.4.

   (b) Add the working solution of the polysaccharide to the mHSA working solution dropwise while stirring on a magnetic stirrer. With a multi-channel pipette add 100 µL of the mHSA-men C mixture to each well of an Immulon 2 plate.

   (c) Seal plates with pressure sensitive film and incubate at +4°C overnight. Plates can be used up to 14 days after preparation.

8.2 Blocking:

1) After overnight incubation, aspirate plate antigen coat contents into a flask containing 5 % (v/v) Gigasept (1 % formaldehyde).

2) Wash plate 5 times with wash buffer using SkanWasher.

3) Blot plate vigorously on absorbent towel.

4) Fill plate wells with 200 µL of 10 m*M* PBS (pH 7.4) containing 5 % NBBS

5) Seal plate and incubate at room temperature for 1 hour.

8.3 Loading plates:

1) Decant blocking buffer from a single plate (do not decant and apply sera to multiple plates as antibody binding to the antigen occurs before double diluting).

2) Blot plate vigorously on absorbent towel.

3) Fill plate wells, except column 12 (used for 1 x qc dilutions) with 100 µL of serum buffer (see: plate template, p. 12).

4) Using a single channel calibrated pipette, add 100 µL of the qc levels to plate wells A12-B12 (HIGH), C12-D12 (MIDDLE), and E12-F12 (LOW).

5) Add 100 µL serum buffer to plate wells G12-H12 as conjugate control wells (Blank).

6) Add 100 µL of the 2 x reference standard to the standard dilution series (see: plate template, p. 12). Avoiding excessive bubble formation, mix the contents of plate wells A1, A2 and A3 5 times (mixing is done by drawing the solution from a well into the pipette and dispensing back into the same well). Using the same multi-channel pipette tips, transfer 100 µL of the contents of plate wells A1, A2 and A3 to plate wells B1, B2 and B3 and mix 5 times. Continue with the same procedure until you complete transferring 100 µL of the contents of plate wells G1, G2 and G3 to plate wells H1, H2 and H3. After mixing the contents of plate wells H1, H2 and H3, discard 100 µL from these wells so that the final volume is 100 µL.

7) The patient sera (4 sera per plate, 8 twofold serial dilutions, in duplicate) are dispensed in the microtitre plate as follows: the first dilution (2x) of the first patient serum is dispensed into plate wells A4 and B4 (100 µL per well). The first dilution of the second, third, and fourth patient sera are dispensed into plate wells C4 and D4, E4 and F4, and G4 and H4, respectively (see plate template). Using a multi-channel pipette with 8 tips, mix (5 times) the contents of plate wells A5-H5 (column 5). Continue the mixing and dilution procedure through plate wells A11-H11. Discard 100 µL of the contents of plate wells A11-H11 to adjust the volume to 100 µL (see: plate template, p. 12).

8) Seal plate and incubate overnight at +4°C.

8.4 Conjugate:

1) Prepare an appropriate amount of human IgG Fc PAN mab at a final dilution of 1:2000 in S/C buffer.

2) Wash each plate 5 times with wash buffer.

3) Blot vigorously plates on absorbent towel.

4) Add 100 µL of diluted conjugate to each well using a multi-channel pipette.

5) Seal and incubate plates at room temperature for 2.5 hours.

8.5 Substrate development:

1) Just prior to the end of the conjugate step, prepare an appropriate amount of substrate solution by adding one TMB tablet to 10 ml of substrate buffer.

2) As previously done for each plate, aspirate contents, wash 5 times, and invert onto absorbent towel.

3) With a multi-channel pipette add 100 µL of freshly prepared substrate to each well of each plate.

4) Incubate plates at room temperature for 30 min.

5) With a multi-channel pipette, add 100 µL of 2 *M* H_2SO_4 to each plate well.

6) Read the OD of each plate well at 450 nm. Blank each plate on the average of the ODs of plate wells G12 and H12.

9. Microtitre plate template:

	1	2	3	4	5	6	7	8	9	10	11	12
A	REF. STD.			PATIENT # 1 - 8 TWOFOLD SERIAL DILUTIONS IN DUPLICATE (A4 & B4, A5 & B5, etc.)								HIGH
B	TWOFOLD SERIAL											HIGH
C	DILUTIONS			PATIENT # 2 - (C4 & D4, C5 & D5, etc.)								MID
D	IN TRIPLICATE											MID
E				PATIENT # 3 - (E4 & F4, E5 & F5, etc.)								LOW
F												LOW
G				PATIENT # 4 - (G4 & H4, G5 & H5, etc.)								BLK
H												BLK

10. Guidelines for the evaluation and interpretation of ELISA data

1) Check:

   (a) for the uniformity of the assay standard reference curve to previous assay standard reference curves and;

   (b) if the assay standard reference curve r^2 value is at least 0.990. Any plate that does not satisfy these checks should be repeated.

2) Quality Control:

   (a) Check: that each dilution of the quality control (high, middle, low) has a within dilution CV ≤15 %;

   (b) that each within assay CV of the dilutions of the quality control is ≤15 %, and;

   (c) that each dilution of the quality control (high, middle, low) as well as the mean of all three dilutions of the quality control is within ± 2 SD of its established mean. Any plate whose quality controls do not satisfy checks (a)-(c) should be repeated. Occasionally a quality control result will be within ± 3 SD of its established mean.

3) Check that each within dilution average OD for an unknown (after subtraction of the average OD of the blank wells) is ≥ the average OD of the blank wells of the assay. If no unknown within dilution average OD (after subtraction of the average OD of the blank wells) is ≥ the average OD of the blank wells of the assay, report the antibody level of that unknown as being less than the lower limit of detectability of the assay.

4) Check that each within dilution average OD for an unknown is ≤95 % of the average OD of the upper limit of the standard reference working curve. Do not accept any within dilution average OD that does not satisfy this check.

5) Check that each within dilution CV for an unknown is ≤15 %:

   (a) if all or the majority of the within dilution calculated data for an unknown have CVs >15 %, the unknown should be reassayed;

   (b) if one of the two results for a particular dilution of an unknown is not calculated while the other is calculated, do not accept either result;

   (c) if only the 1:50 dilution of an unknown is accepted, signify on the report of that unknown that a 1:50 dilution was used and report the 1:50 antibody level for that unknown as its final result;

   (d) if only the 1:3,200 and 1:6,400 dilutions of an unknown are accepted, reassay that unknown at at least a starting final dilution of 1:100.

6) Check that the within assay CV for an unknown is ≤20 %:

   (a) if two or more of the within dilution results for an unknown are accepted, the ODs of the accepted data increase with increasing serum concentration and the within assay CV is >20 %, signify on the report of that unknown that it is nonparallel to the assay standard reference curve and if the initial 1/50 dilution gives a OD of > 0.5 record the median antibody level for that unknown as its final result; if the OD of the initial 1/50 dilution is < 0.5 take the first (1/50) dilution.

(b) if two or more of the within dilution results for an unknown are accepted, the ODs of the accepted data DO NOT increase with increasing serum concentration and the within assay CV is >20 %, reassay that unknown.

7) Check that the O.D. of each of the blank wells is < 0.1. Reassay plate if ≥ 0.1.

11. Data analysis

The ELISA readings will be captured from the Titertek multiskan MCC 340 by Multiscan Auto Read Version 5.0 software and processed by ELISA version 1.07 software package. Data analysis software (provided by CDC, Atlanta, USA) - 4-parameter logistic curve model. (Reference: *J. Clin. Microbiol.* 1991. 29: 1439-1446.) See "Program ELISA - User's Manual". The calibration factor for total IgG antibody in the CDC 1992 control sera = 19.6 EU/ml.

All results, expressed as total IgG antibody to serogroup C polysaccharide in mg/ml, will be entered into MS Office 97 Excel.