Overview of antibody-mediated immunity to *S. pneumoniae:* pneumococcal infections, pneumococcal immunity assessment, and recommendations for IG product evaluation

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Streptococcus pneumoniae strains colonize the nasopharynx and can cause mucosal infections in the upper airway and middle ear, pneumonias, and invasive infections like bacteremia, sepsis, and meningitis. Over 90 serotypes, defined by the structure of their capsular polysaccharides, are known. Twenty-three of these serotypes cause most infections and several of these serotypes can develop antibiotic resistance.

Susceptibility factors that increase the susceptibility to *S. pneumoniae* mucosal and invasive infections include all forms of primary and secondary antibody deficiencies. Many patients affected by one of these deficiencies benefit from the regular administration of human gamma globulin (HGG) preparations.

Donors of plasma units used to prepare HGG have varying concentrations of IgG antibodies against relevant *S. pneumoniae* serotypes. These antibodies are developed in response to colonization and common subclinical infections and by routine vaccination with *S. pneumoniae* polysaccharide vaccines. The presence of an adequate concentration of these protective antibodies against all prevalent serotypes needs to be determined to assure the effectiveness of HGG.

All presently available methods to assess IgG antibodies against *S. pneumoniae* capsular polysaccharides have advantages and pitfalls that are analyzed in this review. *In vitro* testing does not provide a complete or necessarily accurate measurement of the effectiveness of antibodies *in vivo*. For regulatory purposes, caution needs to be used in the interpretation of currently available assays that measure pneumococcal antibody levels. Monitoring *S. pneumoniae* infections in patients treated with HGG and tracing information about HGG lots used to treat these patients should be encouraged.

STREPTOCOCCUS PNEUMONIAE INFECTIONS

Streptococcus pneumoniae is a gram-positive bacteria characteristically identified microscopically as diplococci. Over 90 different serotypes are identified based on the different shapes of their surface capsular polysaccharides. These individual serotypes have differing virulence and ability to develop antibiotic resistance. Infections caused by pneumococci are quite varied. Twenty-three of these serotypes cause the majority of infections at all ages. Dominant infectious serotypes vary according to region[1] and also with age.[2]

S. pneumoniae serotypes are regularly found as colonizing bacteria in the nasopharynx and throat. From there, they can spread to cause mucosal infections like sinusitis and otitis media, and more severe infections, such as lobar pneumonia and invasive infections, including bacteremia, sepsis, and meningitis (Fig. 1). They may also cause a variety of infections in normally sterile sites throughout the body.

ABBREVIATIONS: ELISA = enzyme-linked immunosorbent assay; HGG = human gamma globulin; OPA = opsonophagocytosis; WHO = World Health Organization.

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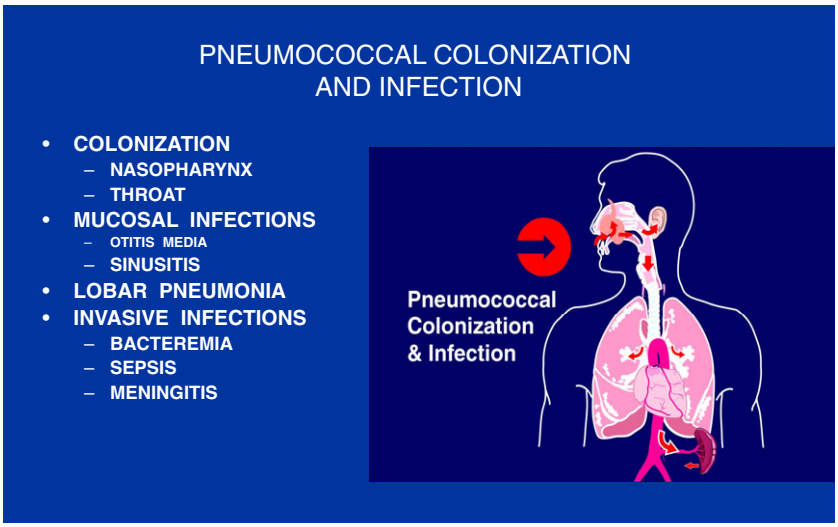

Fig. 1. *S. pneumoniae* colonization, mucosal infections, pneumonia, and invasive infections. The nasopharynx is colonized by different strains of *S. pneumoniae* in addition to other bacteria. From the nasopharynx, pneumococci can invade and cause infections in the sinus cavities, the middle ear, and also in the lungs, which are usually sterile sites. [Color figure can be viewed at wileyonlinelibrary.com]

The outcome of infection with *S. pneumoniae* is influenced by the preexisting levels of specific antibodies, the infecting *S. pneumoniae* serotype, and the site and severity of the infection. The serum concentration of specific antibodies needed to protect against mucosal infections, otitis media, and sinusitis is higher than that required for protection against pneumonia and hematogenous spread of infections like bacteremia, sepsis, and meningitis. This is because of poor antibody and complement penetration to the mucosal infection sites coupled with local structural cofactors that allow the chronicity and recurrence of infections.

S. PNEUMONIAE ANTIGENS

Pneumococci have a unique structure that includes a capsule, a cell membrane, and a polysaccharide cell wall protecting them from complement-mediated lysis. Electron micrography studies have revealed that the capsule is somewhat permeable. Antibody complement and other proteins can reach the cell wall, but phagocytes cannot, and gram-positive bacteria are not lysed by complement because of the thickness and structure of the cell wall, not the failure of complement to penetrate"[3] (Fig. 2). Several *S. pneumoniae* antigens (capsular and noncapsular) induce the development of antibodies. However, only antibodies against capsular antigens induce clearance of pneumococcal infections through phagocytosis by both neutrophils and macrophages. It is the serotype-specific polysaccharide antigens that are targeted by specific antibodies. Each of the polysaccharides of the over 90 pneumococcal serotypes is different (Fig. 3). These capsular polysaccharides are used to prepare *S. pneumoniae* polysaccharide vaccines.

The capsule also contains surface proteins (Fig. 2). There has been some interest in developing anti–capsular protein vaccines, which might be antigenic in the first year of life and may be protective across several different serotypes based on the structure of their surface polysaccharides. The success of conjugate protein–polysaccharide vaccines has slowed the interest in further studying the development

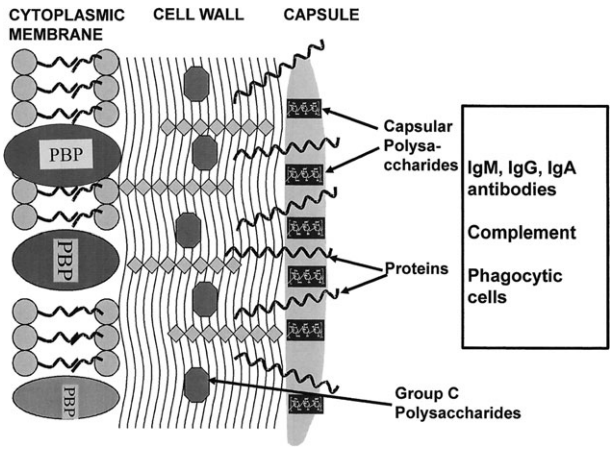

Fig. 2. Structure of the *S. pneumoniae* capsule and cell wall. The capsule contains serotype-specific capsular polysaccharides and also proteins. Group C polysaccharides are part of the cell wall. PBP = protein-binding protein. Further detail about the structure of *S. pneumoniae* can be found in a review by Geno et al.[4] Capsular polysaccharides and surface proteins interact with antibodies, which opsonize whole bacteria. Complement is activated directly and after interaction with IgM and IgG antibodies to augment opsonization. Complement is unable to lyse pneumococci protected by their capsule. Phagocytic and antigen-presenting cells phagocytose pneumococci after opsonization.

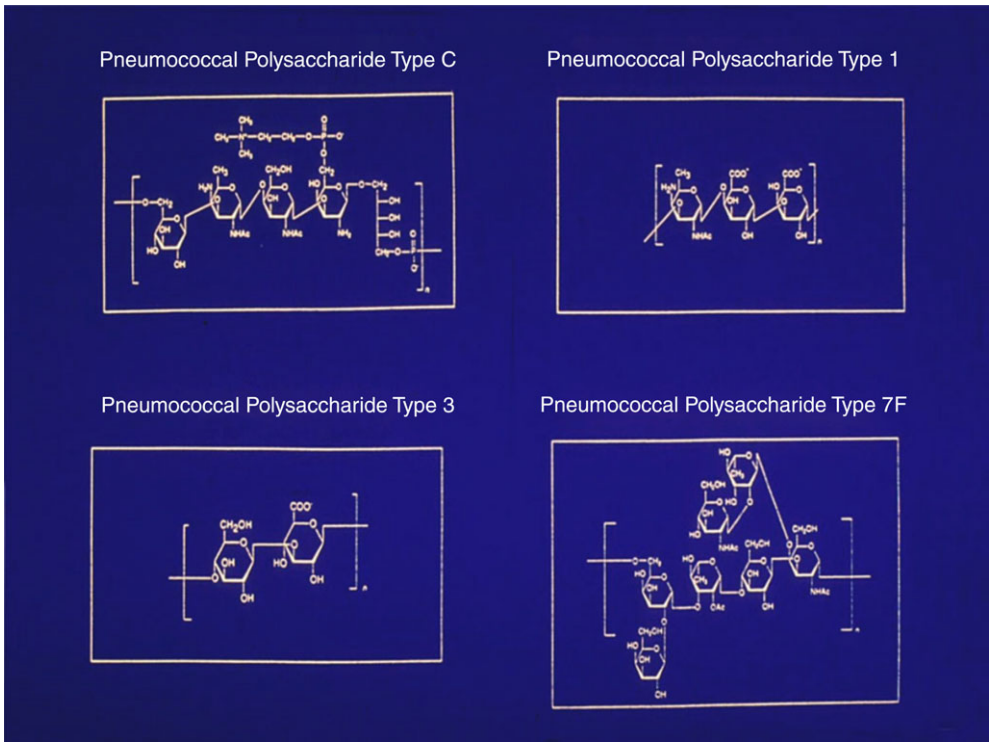

Fig. 3. Chemical structure of polysaccharide C and of three different capsular polysaccharides. Capsular polysaccharides of each of the over 90 *S. pneumoniae* serotypes are structurally and antigenically different and are recognized by serotype-specific antibodies. Capsular polysaccharides that are found in *S. pneumoniae* strains that do develop antibiotic resistance are now included in conjugate vaccines. [Color figure can be viewed at wileyonlinelibrary.com]

of pneumococcal protein vaccines and the potential protective and diagnostic roles of anti-protein antibodies. The proteins used to prepare conjugate polysaccharide vaccines are not derived from *S. pneumoniae.*

When *S. pneumoniae* bacteria are phagocytosed and the antigens are presented to T and B cells, antibodies to both surface and subcapsular antigens are developed. This simultaneous antibody production is directed to antigenic proteins and polysaccharides alike. *S. pneumoniae* subcapsular antigens include polysaccharide C (Fig. 2**)**. Development of antibodies to surface, subcapsular, and cytoplasmic antigens occurs for all bacteria, regardless of the presence of a capsule. Antibodies to antigens that are not exposed on the bacterial surface have no protective value because they do not reach these antigens and therefore cannot enhance opsonization and phagocytosis. Measuring such antibodies may have diagnostic value in some situations because their presence documents a past or present infection, but they do not document severity of infections.[5,6]

IMMUNITY TO *S. PNEUMONIAE*

S. pneumoniae are extracellular pathogens that need to be phagocytosed to be eliminated. Their capsule protects these pathogens against complement-mediated lysis and from T-cell and natural killer cell immunity. Antibodies are therefore the most important form of acquired immunity against these organisms. Antibodies against serotype-specific polysaccharides are found in the IgM, IgA, and IgG fractions. An antibody response to an infection or to a vaccine begins with the development of IgM antibodies. In a normal response, these IgM antibodies are replaced by IgG antibodies that provide long-term protection (Fig. 4). IgG antibodies are found in all IgG subclasses, although anti-polysaccharide antibodies predominate in IgG subclass 2. Variable percentages of the different subclasses, variable antibody content, and different half-life influences in vivo concentration after IgG infusion (Table 1). In circulation, IgM and IgG antibodies are essential, while on mucosal surfaces IgA antibodies provide an important part of protection against *S. pneumoniae* colonization and infections.

Antibodies are essential to trigger bacterial phagocytosis by neutrophils because without antibodies or complement, neutrophils do not recognize pneumococci as a target for phagocytosis. Antibodies alone trigger phagocytosis but bacterial uptake is enhanced by the addition of complement triggered through the classic complement activation pathway by the presence of IgM and IgG antibodies on the bacterial surface. The differential ability of IgG antibodies present in different IgG subclasses can also influence the opsonizing capabilities of anti-pneumococcal polysaccharide antibodies.

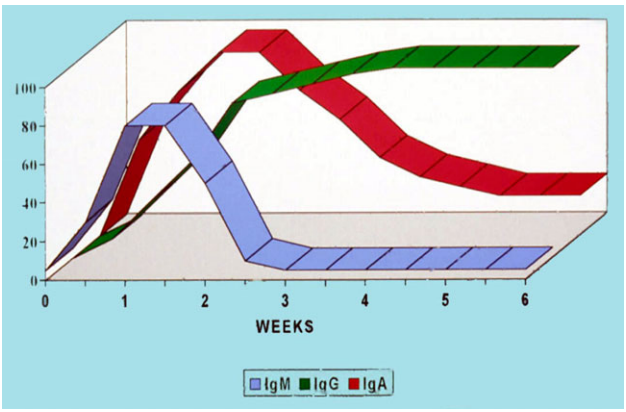

Fig. 4. Development and persistence of IgM, IgA, and IgG antibodies after infections or immunization. After subclinical infections or immunization with pneumococcal vaccines, IgM, IgG, and also IgA antibodies are developed. The antibodies that have the longest half-life and provide long-term protection are antibodies of the IgG class. These have been the object of most assessments of antibody-mediated immunity to *S. pneumoniae* by using reagents that measure only these antibodies. [Color figure can be viewed at wileyonlinelibrary.com]

The concentration of anti-pneumococcal serotype antibodies in the four IgG subclasses was determined by assessing weight-by-volume methods.[7–10] The higher concentration of these antibodies in the IgG2 subclass, known to have somewhat lower complement-activating activity, could mean a lower functional value of these antibodies. However, given that the highest IgG subclass concentration is IgG1 also containing anti–*S.pneumoniae* polysaccharide antibodies, as do the IgG3 and IgG4 fractions, measurements of *S. pneumoniae* antibodies by either enzyme-linked immunosorbent assay (ELISA) or bead-based assays do not routinely differentiate antibodies in the different IgG subclasses. By measuring these IgG antibodies in whole serum or in purified IgG preparations, measuring antibodies in one subclass only would not reflect the true concentration of IgG antibodies.

Clinically, decreases in the concentration of IgG2 correlate with a higher frequency of poor specific antibody responses to pneumococcal polysaccharides and frequently with poor persistence of antibodies elicited by immunization.[11]

TABLE 1. IgG subclass properties

	IgG1	IgG2	IgG3	IgG4
Percentage of total IgG	61	30	5	4
Antibodies to:				
Toxoids	+	+	+	+
Viral antigens	+	+	+++	?
Polysaccharides	+	+++	+	?
Half-life (days)	30	27	16	?

In summary, both the pneumococcal surface antigens and the type and concentration of the antibodies involved must be considered when assessing antibody-mediated immunity to *S. pneumoniae* serotypes. Assessment of the opsonophagocytic qualities of antibodies in the different IgG subclasses for clinical or gamma globulin testing purposes would be very difficult, as it would require IgG subclass purification.

ASSESSMENT OF ANTIBODIES TO *S. PNEUMONIAE* ANTIGENS

Evaluation of immunity

The evaluation of immunity against pneumococci is important in several different areas: evaluation of antibody-mediated immunity in patients suspected of having an antibody immunodeficiency unresponsive to polysaccharide antigens, assessment of specific immunity elicited by pure or conjugate pneumococcal polysaccharide vaccines, and measurement of the antibody content of therapeutic IgG preparations.

Several different methods have been used for these purposes. The most important antibodies that are measured by these assays are IgG antibodies. Most commonly used assays do not differentiate the subclass of IgG involved. Subclass assessment becomes important when assessing the half-life of passively transferred antibodies by transplacental transmission of antibodies to the fetus[12] or when infusing therapeutic IgG preparations. IgM and IgA antibodies can also be measured, but since they do not confer long-lasting systemic immunity, they are not commonly assessed for the purposes listed above.

The specific pneumococcal antigen against which antibodies are measured is an important variable. The antigens used most commonly are individual serotype-specific capsular polysaccharides of pneumococci. The availability of purified serotype-specific polysaccharides and of multivalent vaccines presently containing between 10 and 23 serotype-specific polysaccharides allows the measurement of multiple antibodies against different serotypes. This is important because in different individuals or in different IgG preparations the concentration of specific antibodies against different serotype polysaccharides can vary significantly. An ideal pattern of response is a protective concentration of antibodies to all serotypes measured. In patients, high levels of antibodies to all or most serotype-specific polysaccharides is considered evidence of normal antibody-mediated immunity. Similar criteria can be applied to the assessment of antibody content in therapeutic IgG preparations. It is important to note that high levels of antibodies to only one or a few serotypes may be found together with complete unresponsiveness to other serotypes. Furthermore, the serotypes eliciting a high response vary in different individuals (author's personal observations). It is therefore not surprising that

attempts to identify a response to one or several selected serotypes as representative of the response to all or most serotypes included in the 23-valent polysaccharide vaccine have failed.

Antibodies against C polysaccharides develop after natural infection and need to be absorbed prior to testing for anti–surface polysaccharide. Many serotype-specific antigens used to test antibodies contain polysaccharide C, falsely giving higher antibody concentrations to an individual serotype. Similarly, the preparation of type 22F polysaccharide was found to have a component that is cross-reactive with several other serotype-specific polysaccharides and may give higher antibody measurements against cross-reactive serotypes.[13,14] Both antibodies to polysaccharide C and 22F need to be absorbed prior to any assay measuring serotype-specific antibodies.

Serotype-specific antibody tests

Two main methods are used to measure IgG antibodies against individual polysaccharide serotypes, the ELISA and multiplex assays.

A standardized and reproducible method is the World Health Organization (WHO) ELISA test.[15] This method defines the specific way to perform the ELISA. It was developed under the auspices of the WHO, which invited 22 laboratories to participate in an extensive study to evaluate the IgG antibody response to the serotypes included in the newly developed conjugate polysaccharide vaccines.[12,16] Initially, it measured IgG antibodies against seven serotypes included in the first conjugate vaccine. Since then, the same methodology has been used to measure up to 23 serotypes. Before testing, sera must be absorbed with C-polysaccharide antigen, which induces nonprotective antibodies, and with serotype 22 polysaccharide to remove cross-reactive antibodies. The WHO ELISA results were initially calibrated based on the Food and Drug Administration 89SF standard, now replaced by 007sp, and by laboratory standards prepared using 89SF.

Antibodies measured by ELISA are expressed in micrograms per milliliter (μg/mL). Serotype-specific titers as low as 0.35 μg/mL were deemed protective based on studies of pneumococcal conjugate vaccine in infants and young children showing that vaccine trial subjects reaching this level of antibodies did not have invasive infections with the corresponding *S. pneumoniae* serotype and therefore have been considered to be protective against invasive infections.[7,17–19] Antibody levels that correspond with protection from infection in adults are not as well described.

A titer of 1.3 □g/mL is generally considered protective against mucosal infections and is used as the threshold of response.[20] This concentration is equivalent to 200 ng of antibody nitrogen per milliliter (N/mL) per serotype using a conversion factor of 160 ng of antibody N/ml to 1 μg/mL.[21]

A method that is easier to use including individual antibodies to all 23 serotypes included in the polysaccharide vaccine is also available. It is based on a multiplex fluorescent bead assay (Luminex). Luminex technologies allow simultaneous quantitation of multiple serotype-specific antibodies. It is used by most reference laboratories in the United States. Although the Luminex assay is an attractive alternative to ELISA, its correlation with ELISA test results is not perfect.

The difference of results obtained with the ELISA and Luminex methods was recently documented in a comprehensive comparison of the multiplex Luminex xMAP Pneumo 14 assay with the gold standard WHO ELISA in sera samples from children and adults taken pre- and post-Prevenar (Pfizer, Inc.) and Pneumovax (Merck & Co., Inc.) immunization. They concluded that this assay does not allow reliable evaluation of antibody responses to polysaccharide antigens for the assessment of humoral immune competence.[22–25] In our experience, different laboratories offering Luminex determinations show significant differences in laboratory-to-laboratory results (Table 2).

Different laboratories operating in different regions of the world have established their own ranges of serotype-specific antibody concentrations. Serotype-specific threshold values could be applicable for some defined, uniform populations.[26] However, for values obtained by different commercial and private laboratories this kind of definition of antibody responses is unrealistic, as it is influenced by prevalent infections and pneumococcal immunization practices.[27]

Global tests

While most tests of anti-pneumococcal IgG antibodies measure specific anti-serotype antibodies individually, in many places a global ELISA test that measures antibodies against all 23 serotypes present in the 23-valent vaccine simultaneously is preferred because of its simplicity and low cost.

TABLE 2. Comparison or antibody measurement by WHO ELISA and two different Luminex assays in the same patient serum

S. pneumoniae serotype	Luminex A	Luminex B	WHO ELISA
4	0.5	2.9	0.68
6B	1.3	2.0	0.53
9 V	1.8	2.5	0.44
14	0.9	11.5	6.00
18C	0.5	4.0	0.68
19F	2.8	4.6	1.76
23F	10.7	18.2	0.45
1	0.5	15.6	1.76
3	1.3	4.3	1.79
7F	2.3	4.7	1.88
19A	3.0	6.4	-
9 V	1.8	2.5	0.44
11A	0.3	2.5	0.82
15B	1.5	26.8	3.19
33F	3.0	2.3	2.82

ELISA = enzyme-linked immunosorbent assay; WHO = World Health Organization.

The antigen in this assay is the 23-valent vaccine or a commercially available enzyme immunoassay kit.[28] Results in the normal range for this test can be due to antibodies to most or all included serotypes or by high antibodies to only one serotype. Serotype-specific antibody concentrations are needed for an accurate assessment of antibody-mediated immunity to *S. pneumoniae.*

In some places, a global ELISA test that simultaneously measures antibodies against the combined 23 serotypes present in the 23-valent vaccine is used because of its simplicity and low cost.[29] The shortcomings of this test compound the problems of a global assay with those of a multiplex assay. Therefore, we consider that global tests are not suitable for regulatory purposes.

Methods to assess functional immunity: opsonophagocytosis

Opsonophagocytosis (OPA) measures function of IgG antibodies against surface antigens in purified IgG preparations and IgM, IgG and IgA antibodies in patient sera. The target is whole cell *S. pneumoniae* bacteria that express protein and polysaccharide antigens on its surface (Fig. 2). To eliminate the influence of complement on the assays outcomes, the current protocol for multiplexed OPA includes a heat-inactivation step to prevent the activity of endogenous complement in serum.

OPA assays have undergone significant developments that have improved its availability, its use to assess immunity to many different *S. pneumoniae* serotypes, its standardization and the availability of reference sera.[30–33] (See LaFon and Nahm, Measuring quantity and function of pneumococcal antibodies in immunoglobulin products, this journal).

When applied to the assessment of vaccine responses[34] and therapeutic IgG preparation, OPA has offered a welcome improvement over previous use of experimental animals to test the ability of passively administered human antibodies to prevent lethal infections with a single pneumococcal serotype.[35,36]

ANTIBODY CONCENTRATION AND FUNCTION

The only functional assay of anti-pneumococcal antibodies is OPA. OPA is the best test available, but it is available only in selected places and most commercial laboratories do not offer it. It is now used extensively to evaluate vaccine antigenicity by vaccine manufacturers and probably should also be used to assess therapeutic IgG preparations. Clinically, OPA does not measure exclusively IgG antibodies against polysaccharide antigens (Fig. 2) Antibodies of all immunoglobulin classes against polysaccharide and protein antigens on the surface of intact bacteria influence its results when using serum. If not inactivated, complement provides additional opsonization of bacteria that enhances bacterial phagocytosis. Complement would not play a role in the results of OPA assays using purified IgG preparations, although the effect of these antibodies in vivo probably is also influenced by their complement activation capability.

There is not a strict relationship between the weight-by-volume antibody concentration results by ELISA and OPA.[13] The Centers for Disease Control and Prevention reported a good correlation between ELISA results and OPA performed using serum from adult donors. However, when using sera from elderly donors, ELISA concentrations were relatively higher than the OPA results, suggesting a decrease in the opsonizing capability in the elderly population.[37] Booster immunization of this age group partially corrects this difference. In clinical practice, there are patients who have normal ELISA titers but who improve clinically when given therapeutic IgG, suggesting that the lower function observed in the elderly may also be present in other individuals at an earlier age. To what extent OPA results are different from anti-serotype polysaccharide antibodies due to the presence of other immunoglobulins, antibodies against surface proteins and also complement have not been evaluated in a systematic way. Since IgG2 concentrations are not known to be higher (or lower) in the elderly, this effect is unlikely to be related to the lower complement activation capability of IgG2.

PNEUMOCOCCAL IMMUNITY IN HUMAN GAMMA GLOBULIN

Studies of circulating levels of *S. pneumoniae* IgG antibodies after intravenous therapeutic IgG infusion have been performed by ELISA and by OPA. Testing antibodies by ELISA, Knutsen et al.[38] documented that the expected differences between the average peak level achieved after infusion and at the trough level 4 weeks later was observed, reflecting the half-life of IgG. The key differences of concentrations of specific antibodies observed at the trough levels correlated well with the peak concentration. This suggests that the initial concentration of specific antibodies in an IgG preparation determines the antibody levels achieved in vivo. Interestingly, circulating antibody levels were very similar to antibody concentrations achieved with weekly subcutaneous infusions.[38,39]

Tuerlinckx et al.[40] observed that distribution curves differed between serotypes and showed wide dispersion among patients. In 89% to 100% of patients, antibodies against most serotypes reached trough levels of 0.2 □g/mL, a threshold they considered protective against invasive pneumococcal infection.

When studying OPA indices, Lee et al.[41] observed that variations in the indices of OPA against specific serotypes in different lots of human gamma globulin correlated with OPA activity found in patients after infusion of gamma

globulin. Antibodies against only 2 of 26 serotypes tested were below indices considered protective.Correlations of anti-pneumococcal serotype antibodies and infection with the corresponding *S. pneumoniae* serotype, possibly depending on the type of immunodeficiency being treated, have not been documented in human gamma globulin clinical trials.[42]

SUMMARY AND CONCLUSIONS

In summary, our present *S. pneumoniae* antibody testing ability is poor, and different methods measure different components of what *in vivo* is a complex system of antibody-mediated immunity components. At the present time, the best correlations with effective immunity (freedom from infections) are offered by the WHO ELISA and by OPA, but even these methods have significant limitations that still need to be corrected. In the meantime, any product requirements should be formulated with careful consideration of the limitations of presently available evaluation methods.

The OPA method is already the gold standard to evaluate the response to pneumococcal vaccines in the serum of immunized individuals. As methods and standards for the OPA method are being researched and developed, this should become the method of choice to assess functional antibodies in IgG preparations.

In the evaluation of the antibody response of each patient or also each therapeutic IgG preparation, it is important to assess the response to as many different serotypes as possible to obtain accurate information of the concentrations of anti-pneumococcal polysaccharide IgG antibodies.

CONFLICT OF INTEREST

RUS participates in the CSL-Behring Speaker's Bureau; JDME has a financial relationship with Shire UK, Ltd. through a consulting agreement and speaker fees 2018, and with Octapharma through a consulting agreement and speaker fees 2017/18. Additionally, JDME has a financial relationship with Grifols, which includes attendance expenses for ESID 2017, and both a nonfinancial and a financial relationship with ALK Abello, including speaker fee and attendance at BSACI 2016.

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