

Determination of Antibody to Pneumococcal Polysaccharides with Chromic Chloride-Treated Human Red Blood Cells and Indirect Hemagglutination

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A method is described for the quantitation of serum antibody to type-specific pneumococcal polysaccharide. The method uses highly purified pneumococcal polysaccharide coated onto human O⁺ red blood cells by the chromic chloride technique. Each of 14 pneumococcal polysaccharide types was individually coated onto red blood cells and used to determine the antibody response following primary immunization. The method was found to be sensitive, detecting antibody titer increases of several hundred to a thousand-fold. The presence of high preimmunization antibody titers did not obscure the detection of antibody titer increases. The method detected antibody of both the immunoglobulin M and immunoglobulin G class when quantitated after ultracentrifugation and sucrose density gradient separation. By using serum samples obtained from volunteers immunized with a single pneumococcal polysaccharide, the method was standardized resulting in an ability to compare samples taken at different times and obtained from different sources. The method appears to be simple, reproducible, and inexpensive and can be utilized to determine the antibody response following immunization in large population studies.

Despite the availability of effective antibiotic treatment for pneumococcal disease, a significant morbidity and mortality continues to be observed following pneumococcal infection. This appears to be particularly true in compromised hosts such as patients with Sickle cell disease, young children following splenectomy, individuals on immunosuppressive therapy, and the aged (2, 10). Pneumococcal infection also appears to be a primary cause of bacterial otitis media in infants and children (5). Other problems related to pneumococcal infection have emerged. Recently, relative resistance of pneumococcal organisms to penicillin has been described (4). The extent of these problems has led to renewed interest in the prevention of pneumococcal disease, and studies have been undertaken to determine the effect of immunizing individuals with purified pneumococcal polysaccharide (PPS) vaccines. An important part of these studies is the ability to measure the immunogenicity of the antigen utilized. Methods utilized to measure antibody response

must be sensitive, reproducible, inexpensive, and have the ability to quantitate large numbers of serum samples. In this paper we describe a method which fulfills these criteria and which appears to have widespread application for use in immunization programs involving the administration of pneumococcal polysaccharides.

MATERIALS AND METHODS

Consistent coating of red blood cells with the desired antigen was accomplished by strict adherence to the following method. Human type O, Rh-positive, red blood cells were collected in stoppered vacuum tubes by using liquid ethylenediaminetetraacetic acid (EDTA) as the anticoagulant (Becton-Dickinson Vacutainer tube 4784). The vacuum in the tubes was released just prior to adding the cells from the syringe, to expose the red blood cells to atmospheric oxygen which enhances their antigen-coating potential. The cells were stored in test tubes at 5 C for a minimum of 24 to 48 hr and a maximum of 1 week. For coating, approximately 1.0 ml of mixed cells was removed, placed in a 6-ml glass test tube, and washed five times with 5-ml volumes of normal saline.

Centrifugation was carried out at 3,200 rev/min for 1 min in an Adams Serofuge (Clay Adams, Parsippany, N.J.). After the last saline wash, the supernatant was carefully removed, leaving only the washed, packed red blood cells ready for coating with a specific antigen.

A stock solution of chromic chloride (0.0375 M) was prepared by dissolving a preweighed amount of the powder in double-distilled water. Dilutions of the stock CrCl_3 were prepared and designated as follows: 200 μl of stock CrCl_3 solution plus 1.0 ml of normal saline, labeled $5\times \text{CrCl}_3$; 100 μl of stock CrCl_3 solution plus 1.0 ml of normal saline, labeled $10\times \text{CrCl}_3$; 50 μl of stock CrCl_3 solution plus 1.0 ml of normal saline, labeled $20\times \text{CrCl}_3$ (3, 7, 8).

PPS of various types were supplied by Eli Lilly & Co., Indianapolis, Ind., and by Benjamin Prescott, National Institutes of Health. The PPS antigens were sonically treated into solution (Mettler Ultrasonic Bath) for 5 min in concentrations of 2.0 mg of PPS per ml of normal saline. The antigens could be stored in solution at 5 C for several months.

Varying concentrations of antigen and chromic chloride dilutions were utilized to determine the optimal concentrations of each for coating the red cells (1, 7). It was found that a standard concentration of PPS antigen (2.0 mg/ml) could be used for all types utilized thus far. Slight differences in coating were noted between the Eli Lilly-prepared PPS antigens and those prepared by Benjamin Prescott. Where the Eli Lilly-prepared PPS type XIV failed to coat red cells at a concentration of 4.0 mg of PPS/ml with a $5\times$ dilution of stock CrCl_3 , the PPS type XIV prepared by Benjamin Prescott coated at a concentration of 2.0 mg of PPS/ml with a $5\times$ dilution of stock CrCl_3 solution. The concentration of chromic chloride necessary for coating varied depending on the PPS type being used. That is, where PPS type I might require a $10\times$ dilution of stock CrCl_3 solution for successful coating, PPS type III required a $5\times$ dilution of the CrCl_3 stock solution.

For proper coating of the red cells with antigen, the addition of reagents in the following sequence was found to be essential: (i) 50 μl of washed, packed red blood cells were pipetted into a 6-ml tube (12 by 75 mm), (ii) 100 μl of pneumococcal polysaccharide antigen solution (2.0 mg/ml in normal saline) was then added while gently shaking the tube, (iii) 100 μl of a dilution of the stock solution of chromic chloride was added and the tube was shaken by hand for 4 min. The cells were then washed three times in 5-ml volumes of normal saline followed by spinning at 3,200 rev/min in the Adams Serofuge for 30 sec. After the last wash, the cells were suspended in 3.0 ml of a solution of 0.01 M phosphate-buffered saline (pH 7.2) containing bovine serum albumin and dextrose (100 ml of phosphate-buffered saline, 1.6 ml of 30% bovine serum albumin, and 100 mg of dextrose; designated as solution I). This coated cell suspension could be kept for 5 days at 5 C giving reproducible results when diluted in the ratio of 0.5 ml of cell suspension plus 3.0 ml of solution I.

For large assays, where the serum samples to be

tested against one PPS type exceeded 40, the Autotiter III (Canalco, Inc., Rockville, Md.) was employed. This machine automatically adds 25 μl of diluting solution (solution II: 0.01 M phosphate-buffered saline, pH 7.2, containing 50 μl of Tween 80/100 ml, 1.0 mg of polyvinylpropylene [PVP] per 100 ml, and 1.5 ml of 30% bovine serum albumin per 100 ml) to all but the first row of wells of the Canalco-supplied plates ("Autotray," clear, V bottom). The Autotiter III then serially dilutes the previously applied serum samples in the first row of wells, adds uncoated red cells to the first row of wells as a control for antibodies against blood group substances, and then adds coated red cells to the remaining 14 rows of 8 wells. The plates were sealed with tape, agitated for 200 rev on a TekTator (Scientific Products no. R4318), and incubated in a horizontal position for 2 hr at room temperature. The plates were then centrifuged in special centrifuge carriers (Canalco, Inc., Rockville, Md.) at 1,000 rev/min for 3 min (International model PR-J). After centrifugation, the plates were elevated at a 60° angle for 10 min, and the "run down" pattern was read (9). The highest titer that resulted in agglutination (no run down) was recorded as the end titer.

The Canalco microtiter plates were used directly as supplied, without previous washing. The serum samples were applied with a 50- μl Eppendorf pipette with disposable tips. The same application could be made 24 hr preceding the run (in this case, the plates were briefly agitated just before starting the run on the following day). As many as 50 plates (400 serum samples) could be prepared and stored at 5 C overnight.

The microtiter dilutors on the Autotiter III are spun in a running water bath before starting each new plate. An electric flaming device on the machine is bypassed because of the probability of carbon flecks interfering with the run down pattern. The dilutors are periodically placed in a Mettler ultrasonic bath for 10 min for thorough cleaning.

In assays utilizing the Autotiter III, 0.025 ml of washed, packed red blood cells were coated with the specific antigen with the same ratio of cells-antigen-chromic chloride as noted above. That is, 0.025 ml of washed, packed red cells, 0.50 ml of specific PPS antigen at 2.0 mg/ml of normal saline, and 0.50 ml of the appropriate dilution of stock chromic chloride. In this way, 105 ml of the coated red cells diluted in solution I could be prepared. The cell suspension was placed in a 125-ml Erlenmeyer flask, and constant mild stirring was maintained with a small magnetic stirrer throughout the run. A smaller amount of uncoated red cells was prepared, and this suspension was also stirred throughout the run.

Standard antisera against various pneumococcal polysaccharide types were obtained by immunizing individuals with specific types of PPS (Eli Lilly & Co.) and 2 weeks later drawing one unit of blood. The blood was allowed to clot for 24 hr at 5 C before spinning at 2,500 rev/min for 30 min at 5 C. The serum was fractionated into small tubes and frozen. An average of 100 tubes per standard PPS type were

obtained in this way. It was possible to check the reliability of a particular run on the Autotiter III by including, at various points along the run, an antiserum with a known titer. In addition, each time it was necessary to use a particular standard PPS antiserum, only one tube needed to be thawed. The effect of freezing and thawing on pneumococcal polysaccharide antibody of a particular type was ascertained by taking one standard PPS tube and subjecting it to a recorded number of freezings and thawings. The resultant titer could be compared with that of a standard PPS tube thawed only once.

Selected sera (high titer) were analyzed for antibody following ultracentrifugation in linear 10% to 50% (w/v) sucrose-0.15 M sodium chloride gradients. Samples were spun at 4 C for 14 hr at 34,000 rev/min in a model L Ultracentrifuge (Beckman Instruments, Palo Alto, Calif.) with a Spinco SW39 rotor. Serial samples were obtained by puncturing the bottom of the tube and collecting the drops. Samples were assayed for immunoglobulin G (IgG) and immunoglobulin M (IgM) content by radial diffusion in agar. Samples containing IgG were pooled into a single fraction as well as those containing IgM. Hemagglutination titers were determined on pooled samples.

Cord blood samples were assayed for "background" antibody to pneumococcal polysaccharide. Any antibody detected would have to represent passive transfer and hence would be primarily of the IgG class. Cord immunoglobulins were assayed by radial diffusion in agar.

Selected sera were studied by both the indirect hemagglutination method and radioimmunoassay by using ¹⁴C-labeled polysaccharide (performed by Robert Douglas and Robert Austrian). There was agreement in the detection of antibody response (details will be the subject of a future report).

2-Mercaptoethanolamine (0.2 M) was added in equal volumes to selected sera and incubated for 12 hr at 4 C. Antibody titers were performed on these samples as well as samples diluted with equal volumes of 0.01 M phosphate-buffered saline, pH 7.2.

RESULTS

A total of 14 pneumococcal polysaccharide types have been successfully coated onto red blood cells. Table 1 lists these types, their origin, and the concentrations of CrCl₃ necessary for successful coating. These concentrations were derived utilizing a single donor of red blood cells. The concentration of CrCl₃ necessary for optimal coating may vary with the use of different donor red blood cells.

Table 2 shows the antibody response of three patients (hexavalent vaccine, Eli Lilly & Co., Indianapolis, Ind.) after immunization with 0.05 mg of each of six pneumococcal polysaccharides given subcutaneously. Postimmunization samples were drawn 2 weeks after immunization. The sera were then tested with the six

pneumococcal polysaccharide types comprising the hexavalent vaccine, and the titers were compared with those of the preimmunization serum samples. Results expressed as "fold increase" indicate only the increase or decrease. A decrease in antibody titer (a postimmunization titer less than preimmunization) is ex-

TABLE 1. *Pneumococcal polysaccharide types coated*

PPS type ^a	PPS origin	CrCl ₃ dilution required
I	B. Prescott ^b	10×
II	Eli Lilly & Co. ^c	10×
III	Eli Lilly & Co.	5×
IV	Eli Lilly & Co.	10×
V	Eli Lilly & Co.	5×
VI	B. Prescott	5×
VII	Eli Lilly & Co.	10×
VIII	Eli Lilly & Co.	10×
IX	Eli Lilly & Co.	5×
XII	Eli Lilly & Co.	10×
XIV	B. Prescott	5×
XVIII	Eli Lilly & Co.	10×
XIX	Eli Lilly & Co.	10×
XXIII	B. Prescott	10×

^a PPS concentration 2.0 mg/ml in normal saline.

^b National Institutes of Health, Bethesda, Md.

^c Indianapolis, Ind.

TABLE 2. *Immunizations with hexavalent vaccine^a*

Patient	PPS type	Preimmunization ^b titer	Postimmunization titer	Fold increase
A	I	0	0	0
	III	8	64	8
	IV	512	512	0
	VII	64	256	4
	VIII	64	1,024	16
	XII	64	256	4
B	I	0	4,096	4,096
	III	128	128	0
	IV	512	2,048	4
	VII	512	512	0
	VIII	256	>8,192	>32
	XII	>8,192 ^c	>8,192	0
C	I	0	1,024	1,024
	III	16	256	16
	IV	512	1,024	2
	VII	512	512	0
	VIII	4,096	>8,192	>2
	XII	128	2,048	16

^a Eli Lilly & Co., hexavalent vaccine.

^b Expressed as reciprocal of dilution.

^c Maximum titer read on plates.

pressed as the fold change with a negative sign. None were recorded in this series. Antibody titer increase was expressed as fold increase to reduce variations in expression of results. The highest titers which could be read without further dilution of sera was 1:8, 192.

The results in Table 3 show the antibody response of patients of different ages to a monovalent vaccine (Eli Lilly pneumococcal polysaccharide type I, 0.05 mg, subcutaneously) after a 2-week postimmunization period.

Table 4 shows the antibody response of four patients immunized against two different monovalent types of pneumococcal polysaccharide (Eli Lilly pneumococcal polysaccharide type III and type VIII, 0.05 mg of each, given subcutaneously) with postimmunization samples drawn at 2 weeks, 6 months, and 1 year. Antibody titer increases persisted up to 1 year following immunization without additional known antigenic stimulation.

Table 5 illustrates the results of antibody determination performed on selected sera following sucrose density ultracentrifugation. Antibody of both the IgG and IgM class was detected in significant titers 5 days and 7 days

TABLE 3. Immunization with Eli Lilly pneumococcal polysaccharide type I

Patient	Age	Preimmunization titer	Postimmunization titer	Fold increase
D	Adult	64	256	4
E	11 years	16	256	16
F	7 years	16	32	2
G	4 years	32	64	2
H	18 weeks	0	32	32

TABLE 4. Long-term immunization study

Patient	PPS type ^a	Preimmunization titer	Fold increase		
			2 weeks	6 months	1 year
J	III	2	32	8	8
	VIII	8	8	4	4
K	III	8	16	4	4
	VIII	16	2	0	0
L	III	8	16	4	4
	VIII	8	8	4	4
M	III	4	32	2	2
	VIII	4	16	2	2

^a Eli Lilly & Co., Indianapolis, Ind.

TABLE 5. Quantitation of IgM and IgG antibody following ultracentrifugation and sucrose density separation^a

Patient	PPS II				PPS III			
	Day 5 ^b		Day 7		Day 5		Day 7	
	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM
N	2	4	4	4	128	32	128	16
P	64	64	2	16	8	16	0	128
Q	128	128	256	128	128	16	128	4

^a Expressed as reciprocal of titer.

^b Number of days following immunization.

TABLE 6. Quantitation of pneumococcal antibody in cord blood

Patient	PPS type III	PPS type VIII	Milligrams/100 ml		
			IgG	IgM	IgA
R	32	256	900	0	107
S	4	16	1,460	13	0

TABLE 7. 2-Mercaptoethanolamine (ME) inactivation of antibody

Patient	Titer before 2 ME inactivation	Titer after 2 ME inactivation
T	128	2
U	32	8
V	512	512

after immunization.

Cord blood samples assayed for background antibody to pneumococcal polysaccharide were found in two instances to have significant antibody titers to polysaccharide type III and type VIII (Table 6). Since cord blood antibody represents passive transfer of maternal antibody, detection of antibody in cord serum must represent primarily antibody of the IgG class. Quantitation of serum immunoglobulins revealed IgM absent in the infant with the highest titer of pneumococcal polysaccharide and IgA absent in the other infant.

2-Mercaptoethanolamine inactivation of antibody on three sera (Table 7) resulted in a decrease in titer in two. One sample decreased from a titer of 128 to 2. In another sample, the titer remained unchanged. These results were interpreted as indirect evidence of inactivation of IgM antibody and of some IgG antibody detection by the indirect hemagglutination method.

DISCUSSION

A total of 14 pneumococcal polysaccharide types were individually coated onto human O+ red blood cells. The use of human cells eliminated the difficulty of heterophil antibodies frequently found when sheep red blood cells were used. No difficulty was encountered in coating the polysaccharides except for the occasional requirement of a higher chromic chloride concentration (Table 1).

The method detected antibody titer increases in patients with no preimmunization titers and high preimmunization titers (Table 2). The antibody response of adults as well as infants and children was significant and did not appear to be related to the presence or absence of a preimmunization titer (Table 3). Significant antibody titers (expressed as fold increase compared to the original preimmunization sample) were found up to 1 year after immunization (Table 4).

Antibody of both the IgG and IgM class was detected. Table 5 shows antibody titers on sera following ultracentrifugation demonstrating antibody in both IgG and IgM fractions. Table 6 demonstrates the presence of significant antibody to pneumococcal polysaccharide in the cord blood of two infants; one without serum IgM and one without serum IgA. Since passive transfer of maternal antibody to the infant resides in IgG, these results offer further confirmatory evidence that the indirect hemagglutination method detects antibody of both the IgG and IgM class. The detection of antibody following 2-mercaptoethanolamine inactivation also confirms these observations.

Standardization of the method was accomplished by several means. Normal individuals were immunized with a single pneumococcal polysaccharide. A unit of blood was obtained 14 days later, and the serum was fractionated into 1-ml samples which were subsequently frozen and stored. Each run of antibody titers subsequently included a standard and the results of the antibody titer compared with previous runs. Comparison of results was made by calculating fold increase rather than actual antibody titer. For example, a patient with a preimmunization titer of 1:8 and a postimmunization titer of 1:64 and another patient with a preimmunization titer of 1:256 and a postimmunization titer of 1:2,048 would both have a fold increase of 8. It was felt that the use of fold increase would also decrease errors in comparison due to minor variations in the sensitivity of the assay. If, for example, the previous illustration represented

different results obtained on the same patient run on different days, then the final result would be identical, e.g., fold increase of 8, even though the actual titers varied.

Difficulties involving consistent coating were overcome by using the same donor of red blood cells. Blood was drawn each Friday and then used during the following week.

Large numbers of serum samples could be quantitated by using a semi-automated hemagglutination device (Autotiter III, Canalco Inc.). To avoid diluting serum samples, the sensitivity of the method was reduced so that the highest positive titer was less than 1:8,192. This was accomplished by increasing the coated red blood cell concentration. Reduction in sensitivity did not affect results when expressed as fold increase.

Repeated freezing and thawing or prolonged storage of serum samples at -20°C , or both, did not significantly alter hemagglutination titers.

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