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Spin Membrane Immunoassay for Use in Meningococcal Serology

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A modified and improved spin membrane immunoassay has been developed for detecting complement-activating antibodies to *Neisseria meningitidis* capsular polysaccharide antigens. The polysaccharides were incorporated in the membranes of large unilamellar vesicles prepared by the reverse-phase evaporation method and filled with the water-soluble spin label tempocholine chloride. Upon addition of group-specific antisera and complement, the lipid membrane was damaged and the spin label leaked out. This process was monitored by electron spin resonance spectroscopy. A satisfactory assay was developed for polysaccharides of group A and C, whereas in the case of the B system the assay was more labile. The method is rapid and has a sensitivity comparable to that of radioimmunoassay. When studying paired sera from five recruits vaccinated with an A+C polysaccharide vaccine, significant rises in titers to both A and C polysaccharides were observed in all the postvaccination sera.

During the last decade there has been rapid progress in the development of appropriate vaccines for bacterial meningitis. Based on the initial efforts of Goldschneider et al. (8, 9) and Gotschlich et al. (11, 12), highly purified high-molecular-weight capsular polysaccharide vaccines have been developed and successfully used for the prevention of disease due to infections of *Neisseria meningitidis* of the serogroups A and C. In contrast, B polysaccharide has been found to be nonimmunogenic in humans (29). Recently, vaccines composed of noncovalent complexes of B polysaccharides and lipopolysaccharide-depleted outer membrane material from group B meningococci have been shown to give immune responses in vaccinees (33).

The effect of a vaccine has to be evaluated by some serological assay before it may be tested for protection. For meningococcal antibodies, the most important methods are the bactericidal activity test (BCA) (7, 8), the passive hemagglutination test (2), the radioimmunoassay (RIA) (17) or solid phase RIA (30), and the enzyme-linked immunosorbent assay (7).

A problem when comparing the results of the different tests is that they do not measure the same parameters. Some measure antibodies against the polysaccharides only, others against outer membrane proteins as well. In some tests, immunoglobulin G (IgG) dominates the response, in others, IgM dominates. Hence, the different methods often given complementary information. Furthermore, the sensitivity, the convenience in use, and the time needed for the analysis vary from test to test. Although the RIA and solid phase RIA tests are rapid and sensitive methods for quantitating A and C polysaccharide-binding antibodies, no good test has been developed for anti-B antibodies or exclusively for complement-activating antipolysaccharide antibodies when the antigen is located in a membraneous environment. Attempts to develop a complement-fixation test with purified polysaccharide antigens have met with failure (2). The BCA assay is assumed to be the best measure of the immune status of an individual. The test is, however, both time and reagent consuming, and it is difficult to standardize the assay. The results will not tell us about specificity of the antibodies nor exactly against what antigens they are directed. A sensitive and rapid assay for detecting complement-activating antibodies against the group-specific polysaccharides would therefore be of great clinical and theoretical value.

The work of Gotschlich et al. (10) made it clear that the capsular polysaccharides from the meningococci have a hydrophobic 1,2-diacylglycerol end. This should, in principle, enable these substances to be incorporated into or attached to the membranes of artificial vesicles or liposomes produced from phospholipids. By adding specific antibodies and complement to polysaccharide-sensitized liposomes filled with a marker substance, immune lysis can be utilized in an assay for complement-activating antibodies

analogous to the BCA test.

Kinsky and co-workers (18) developed the first immunoassay based on immune lysis of liposomes filled with glucose. Later, Humphries and McConnell (15) used a spin label as marker to obtain a more sensitive system. The basic idea in the spin membrane immunoassay (SMIA) is to load vesicles with spin label in high concentration. The exchange interaction broadens and decreases the amplitude of the electron spin resonance (ESR) absorption lines. When the spin label is released from the vesicles, the local concentration, and thus the exchange interaction, is markedly reduced. The absorption lines then become narrow and the amplitude greatly enhanced. Unlike the RIA method, SMIA does not require separation procedures and involves no radiation hazard. It has the advantage of speed, the possibility of full automation, simplicity, and a small sample volume (30 μl). (See for review references 5, 25, and, for examples of applications, references 2a, 4, 14, 15, 23, and 27).

In this work, we present a modified and improved SMIA technique developed for detecting antibodies to meningococcal capsular polysaccharide antigens.

MATERIALS AND METHODS

Lipids. Egg yolk phosphatidylcholine was purchased from Avanti Biochemicals Inc., Birmingham, Ala., and cholesterol and L-α-dipalmitoyl phosphatidic acid were from Sigma Chemical Co., St. Louis, Mo.

Complement. Serum from 4-week-old rabbits which were shown to be nonbactericidal in BCA tests, were used as a source of complement. It was stored in small samples at −25°C for a maximum of 3 months and thawed just before use.

Antisera. Meningococcal agglutinating sera, containing rabbit antibodies against *N. meningitidis* group A, B, or C, which had been adsorbed as necessary to render them group specific in agglutination tests, were purchased from Wellcome Reagents Ltd., Beckenham, England. Also, a human serum, taken 3 weeks after immunization with a Men A+C capsular polysaccharide vaccine, was used as a reference serum. This serum (the HK serum) was kindly given to us by Helena Käyhty, Central Public Health Laboratory, Helsinki, Finland. It contained 2 μg of anti-A antibodies per ml and 21 μg of anti-C antibodies per ml as determined by Käyhty by radioimmunoassay (17).

As a reference in the group B system, we also used a mouse ascites fluid (2-1-B) containing monoclonal antibodies of the IgM class directed against the B capsular polysaccharide from meningococci (30). The concentration of IgM was about 5 mg/ml, estimated from agarose gel electrophoresis. This sample was a gift from W. D. Zollinger, Walter Reed Army Institute of Research, Washington, D.C.

As a test of our method with "clinical samples", we also analyzed sera from five adults vaccinated with the Merieux A+C vaccine (50 μg of each polysaccharide stabilized by 1 mg of lactose dissolved in 0.5 ml of physiological saline). Sera from five volunteers vaccinated with a combined capsular B polysaccharide and type 2 outer membrane protein vaccine were also analyzed. One such vaccine dose contained 50 μg of group B polysaccharide, 50 μg of outer membrane protein, 3 mg of lactose in 0.01% Thimerosal–0.85% sodium chloride in 0.5 ml. This vaccine had been prepared by C. E. Frasch, Office of Biologics, Food and Drug Administration, Bethesda, Md. The A+C sera had been collected at 0, 4, and 20 weeks after vaccination, as part of a vaccination program for military recruits. The samples were collected by K. Bøvre and co-workers, Kaptein W. Wilhelmsen og Frues Bakteriologiske Institutt, University of Oslo, Rikshospitalet, Norway. The B sera were taken at 0 and 2 weeks after vaccination as part of a meningococcal group B vaccine trial (L. O. Frøholm, B. P. Berdal, K. Bøvre, P. Gaustad, A. Harboe, E. Holten, E. A. Høyby, A. Lystad, T. Omland, and C. E. Frash, VIII Int. Congr. Infect. Parasitic Dis., Stockholm, Sweden, abstr. no. 11:8, 1982). All vaccination sera were heat inactivated for 30 min at 56°C before use.

Polysaccharides. As a source of capsular polysaccharides from *N. meningitidis*, we used a meningococcal group A polysaccharide vaccine stabilized by lactose, supplied from Institut Merieux, Lyon, France (Fab. V1107) and a Men A+C vaccine (also stabilized by lactose) from Connaught Laboratories Inc., Cherry Hill, N.J. (Lot 2392 KF). Polysaccharides of group B and C were gifts from W. A. Hankins, Connaught Laboratories, Swiftwater, Pa. These latter preparations were not stabilized by lactose but were stored at −25°C.

Preparation of vesicles. Large unilamellar vesicles were made by the reverse-phase evaporation technique (24) with small modifications. The lipid composition was phosphatidylcholine-cholesterol-L-α-dipalmitoylphosphatidic acid (60:35:5) (mole fractions), and typically, 10 μmol of lipid and 1 ml of aqueous phase were used. The aqueous phase was a mixture of 400 μl of 150 mM spin label tempocholine chloride, prepared as described in reference 19, 400 μl of iso-osmolar veronal buffer, and 200 μl of water. The polysaccharides were added to the aqueous phase from which it becomes incorporated in the membrane. The iso-osmolar veronal buffer was made of 145 mM NaCl, 3.1 mM diemal, 0.97 mM sodium diemal, 0.83 mM $MgCl_2$ and 0.19 mM $CaCl_2$, and the pH was 7.2. A hyperosmolar buffer was made in the same way, but with 1.5 times the concentration used in the iso-osmolar buffer. The vesicles were washed for untrapped spin label either by dialysis or by passing the preparation through a Sepharose CL-2B column equilibrated with the iso-osmolar veronal buffer. The reproducibility of the unilamellar vesicle preparations, as judged from the trapping efficiency (volume per mole of lipid) was relatively high (standard deviation of 20 to 30%). The vesicles were stored at room temperature and diluted extensively before use in the assay. Typically, in the final test, the lipid concentration was 20 nmol of lipid per ml.

SMIA. A 30-μl amount of antiserum diluted in veronal buffer was mixed with 10 μl of the hyperosmolar veronal buffer. To this solution, 10 μl of vesicles, diluted in isotonic veronal buffer, were added followed by 10 μl of complement solution. The sample was incubated under shaking at 37°C for 10 min, taken up in 50-μl capillary tubes, sealed with Sigillum wax

(Modulohm, Denmark), and incubated for 10 min more at room temperature before ESR recording. Alternatively, the incubation was for 20 min at 37°C, directly followed by ESR recording at room temperature. The two different incubation schemes gave identical results.

ESR measurements. The ESR signal intensity was recorded on a Bruker ER-200-D ESR spectrometer equipped with a rectangular cavity. The modulation amplitude and frequency were 1.25 G and 100 kHz, respectively, and a microwave power of 20 mW was used. The capillary with the sample was placed in a 3-mm outer diameter quarts tube, and the solution completely filled the part of the capillary tube inside the cavity. The amplitude of the midfield line was determined by using the g-value controlled device as previously described (26, 28). The amplitude could also be determined in the conventional way (23), but with reduced efficiency and accuracy.

RESULTS

Background release. The absolute amount of spin label released from the vesicles was determined basically as described before (23). Thus, a sample in which buffer was exchanged for antiserum and complement gave, by definition, 0% release. The value obtained by adding Triton X-100 (final concentration 2%) was defined as 100% release. A linear dose-response curve between these two limits was assumed. The background release of spin label due to serum or complement-induced nonimmune-specific lysis was minor. During the incubation it typically was only a few percent.

It was found that the phospholipid composition of the vesicles influenced markedly the background release. Vesicles in which phosphatidylserine or dicetyl-phosphate was the negatively charged lipid instead of phosphatidic acid often possessed an enhanced nonspecific lysis. As also observed by Allen and Cleland (1), the nonspecific lysis of vesicles induced by serum or complement was higher if the hyperosmotic buffer was not included in the reaction mixture.

In spite of efforts in choosing the optimal phospholipid composition and osmotic conditions, the nonspecific lysis and the "natural leakage" from vesicles with polysaccharides in the membrane was higher than for our previously tested systems (22, 23). Thus, vesicles typically had to be used within 8 h after they were removed from the dialysis bag and were generally unsuitable after 3 days from the first washing procedure. Because of leakage and nonspecific lysis, the amplification factor in ESR amplitudes for a positive serum response relative to a negative serum response was only a factor of 2 to 10 but was sufficient to detect a positive response.

Immune lysis. When capsular polysaccharides were incorporated in the vesicles, the addition of group-specific antipolysaccharide antibodies and rabbit complement induced lysis of the vesicles, as monitored by a rapid increase in the ESR amplitude. The lytic reaction followed the same kinetics as observed in our previous systems (23). The reaction was complete after 30 min at room temperature or 10 min at 37°C. No significant lysis beyond the nonspecific one discussed above was observed unless all of the following three components were present: antiserum (antibodies), active complement, and polysaccharides incorporated in the membrane (Fig. 1).

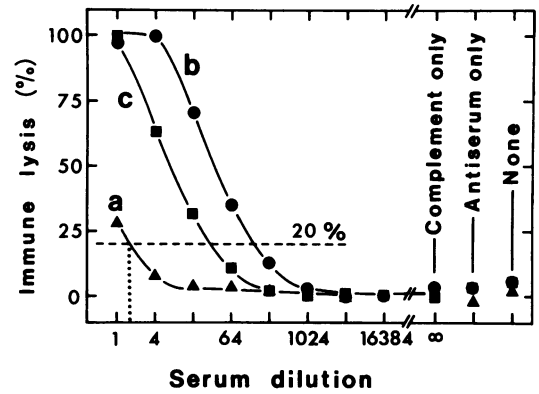

FIG. 1. Effect of vaccination on the antibody titer. The vaccine was a combined group A+C polysaccharide vaccine, and the test system was for anti-A antibodies. Sera a, b, and c were taken at 0, 4, and 20 weeks after vaccination, respectively. The 20% immune lysis line gives the titer value (see text).

With a constant amount of complement per sample, the maximum immune lysis when titrating an antiserum was apparently independent of the actual antiserum. This maximum immune lysis was defined as 100% immune lysis and corresponded to 40 to 80% of the Triton X-100 release of spin label. The 0% immune lysis was defined as the degree of lysis after incubation with only complement and buffer. Again, a linear relationship between ESR amplitudes and the corresponding percent immune lysis was adopted between the 0 and 100% limits.

At the polysaccharide concentrations tested (6 to 25 μg of polysaccharide per μmol of lipid), the degree of lysis and the sensitivity of the test were fairly independent of the antigen concentration.

Group-specific rabbit antisera (meningococcal agglutinating sera from Wellcome Laboratories Ltd.) were tested for cross-reactivity. For most combinations of antisera and vesicles, the cross-reactivity between the A, B, and C systems was very low. Typically, the titer for one group-specific antiserum dropped by a factor of 100 or more when its own group polysaccharide was replaced by that of another serogroup. The only exception from this was the anti-C antiserum

that showed a titer for the B test system of about 1/10 of the titer for the C test system. On the other hand, with the monoclonal anti-B antibodies and the C test liposomes, the titer was reduced to about 1/100 of the titer with the B liposomes.

The release of spin label versus antibody concentration for the group A test system is given in Fig. 2. The results corresponding to two different complement concentrations are shown, and the lower concentration gave a typical prozone. For the higher complement concentration, the immune lysis showed a plateau followed by a fall at a higher antibody dilution.

The shapes of the antibody dilution curves for the A, B, and C test systems appeared to be similar when antisera from the same species were compared. Strong and weak antisera differed mainly by horizontal displacements of the curves (Fig. 1). To indicate the relative strength of the antisera, the serum dilution corresponding to 20% immune lysis is defined as the titer of that serum. The procedure is illustrated in Fig. 1 (the dotted line gives the titer value).

Liposomes prepared with A+C polysaccharides behaved very much like the pure A system, but responded now to both anti-A and anti-C antibodies. The pure group C polysaccharide-coated liposomes also responded very similarly to the above-mentioned systems. The B polysaccharide system, however, tended to give less reproducible results. The maximum lysis was somewhat lower (40 to 60% of Triton X-100 release) and varied considerably for different preparations of liposomes and different antisera. With the group B polysaccharides, the test system was also degraded by time. About 30 to 40 h after the vesicles were made, the percent immune lysis suddenly began to decrease from the maximum value to nearly zero within a few hours. This effect was also observed to some degree for the liposomes made from group C polysaccharides but not from those prepared from the lactose-stabilized vaccines.

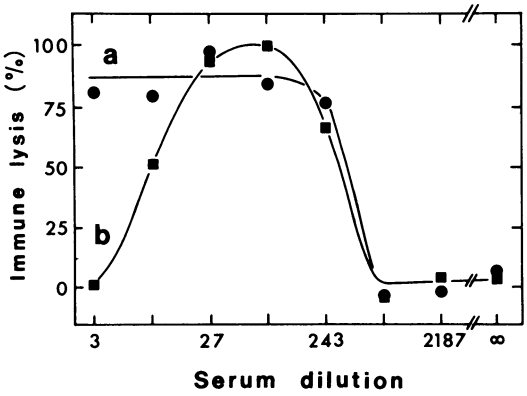

FIG. 2. Immune lysis of group A polysaccharide vesicles when a group A-specific rabbit antiserum and complement were added to the vesicles. In a, 10 μl of undiluted complement was used, whereas in b only 2.5 μl was used. The prozone in b is discussed in the text.

TABLE 1. Titer values determined by the SMIA assay with various liposome test systems[a]

Vaccinee no.	Liposome	0 wk	4 wk	20 wk
1	A+C	2	150	30
2	A+C	450	1,350	1,020
3	A+C	30	210	140
4	A+C	1	130	220
5	A+C	ND	ND	ND
1	A	1	80	20
2	A	830	1,350	1,550
3	A	60	180	180
4	A	5	90	50
5	A	10	240	210
1	C	2	250	80
2	C	100	1,020	390
3	C	1	310	200
4	C	1	170	310
5	C	320	5,040	1,780

[a] The titers are given as the reciprocal of the dilution giving 20% immune lysis. The sera were from vaccinees receiving the Men A+C vaccine. ND, Not determined.

Sensitivity. By using the quantitation procedure discussed above, the HK reference serum had a titer of about 2,000 in the group A polysaccharide system and about 7,000 in the C system. With a concentration of 2 μg/ml for anti-A antibodies and 21 μg/ml for anti-C antibodies (determined by RIA by H. Käyhty), the present tests apparently have a sensitivity limit of about 1 to 3 ng of antibodies per ml.

The sensitivity of the B system was analyzed in the same way as for A and C. As reference, we used the monoclonal mouse antibodies (2-1-B) from W. D. Zollinger. The concentration of anti-B polysaccharide antibodies was about 5 mg of IgM per ml. This sample had a titer of 50,000, indicating a sensitivity limit in this system of around 100 ng of IgM per ml.

Vaccination sera. The sera from five persons vaccinated with the Men A+C vaccine were tested both with A+C liposomes and with A and C liposomes separately. Fourfold titration series in veronal buffer were made, and the titers were determined as described above. The results are given in Table 1.

In the prevaccination sera, three vaccinees (numbers 1, 3, and 4) had relatively low titers in all systems, and two of them had significantly higher values. These high values were mainly caused by anti-A antibodies in case no. 2 and

anti-C antibodies in case no. 5. In the postvaccination sera, we found that all had responded significantly to the vaccine, both with anti-A and anti-C antibodies. After 20 weeks, there was a drop in antibody titers, most obvious for the anti-C antibodies.

When the paired sera from the five vaccinees who had received the combined group B polysaccharide and type 2 outer membrane protein vaccine were analyzed with the B system, we could not find significant (more than twofold) rise in titers against B polysaccharides for any of the vaccinees. However, the instability of the B system discussed above made it difficult to be sure about such small changes in the titer. It should also be mentioned that by using the enzyme-linked immunosorbent assay technique, significant increases of antibodies against outer membrane antigen from a noncapsular variant of a type 2, group B meningococcus was found in all of the five postvaccination sera.

DISCUSSION

The present immunological technique is the only one developed so far which tests for complement-activating antibodies against isolated meningococcal capsular polysaccharides incorporated in a lipid membrane. The immunoglobulins detected are of both IgG and IgM class. Since we have not compared our results with the results from other methods (hemagglutination assay, RIA, enzyme-linked immunosorbent assay, BCA), we can not draw conclusions about the correlation between the various methods. However, hemagglutination assay detects mainly IgM antibodies (2), whereas the BCA test preferentially measures IgG antibodies (11), and by enzyme-linked immunosorbent assay and RIA, we can make the test class specific. Although there is shown to be a correlation between hemagglutination assay, RIA, and BCA antibodies against group A capsular polysaccharides (16), the only test that has been successfully correlated with immunity, and therefore can be used for determination of the immune status, is the BCA test. Probably our SMIA assay is similar to the BCA test, and in addition it has the advantage of being able to study separately the different antigens. To get a full understanding of the immunological response by meningococcus vaccination, both classes and subclasses of the antibodies as well as opsonins and antibody-dependent cellular cytotoxicity should be analyzed.

The assay worked very satisfactorily with group A and C polysaccharides, whereas with group B polysaccharides the system was instable. The reason for the instability of the B system may either be due to the particular sample of B polysaccharide that was available or it may be a general feature of this antigen. It is reported that the B polysaccharides are much more acid labile than the other groups of capsular polysaccharides (29) and spontaneously forms internal esters below pH 6 (20). The instability might also be related to some degradation of the polysaccharides when lyophilized without stabilizer. The C test system, prepared from polysaccharides without lactose, also tended to be degraded over time, although not to the same degree as the B system. This observation might be of interest in connection with an attempted production of a B polysaccharide vaccine. It should be noted, however, that the sudden drop in immune lysis observed did not seem to occur to the same degree and time for all sera.

Apparently, the sensitivity, given in micrograms per milliliter, was 30 to 100 times better for the A and C systems than for the B system, but since the anti-B antibodies available were pure IgM, whereas the anti-A and anti-C antibodies presumably were mainly IgG molecules, this difference is lower on a molar basis.

It should be pointed out here that the titer is only indirectly related to the absolute amount of the particular antibody per milliliter. However, the titer might be an equally good way, if not better, for giving a physiological measure of the immune potential, since now both the number and the avidity of the antibodies influence the result (6).

The difference in sensitivity between the A, C, and B systems probably reflects differences in avidity and binding constants between anti-C and anti-B antibodies. Mandrell and Zollinger (21) have found that at 37°C, both "natural" human anti-C and mouse monoclonal anti-C antibodies had binding constants 20 to 30 times higher than human anti-B or mouse monoclonal anti-B antibodies. In their experiments, they used the same monoclonal anti-B antibody as we did (2-1-B). In the BCA test, human anti-B antibodies require rabbit complement to be bactericidal, whereas with human complement only antibodies against noncapsular antigens were bactericidal (31). Thus, the problems with the B system in SMIA may suggest some interaction between rabbit complement and whole bacteria in the BCA test.

In analyzing the relative strength of different antisera, we observed also that sera with similar titers following our definition, like the rabbit anti-A serum (WA) and the human anti A+C serum (HK), gave distinctly different titration curves with A polysaccharide-coated liposomes (Fig. 3). The same effect was observed for the monoclonal mouse anti-B antibodies and the rabbit anti-B sera with B polysaccharide-coated liposomes.

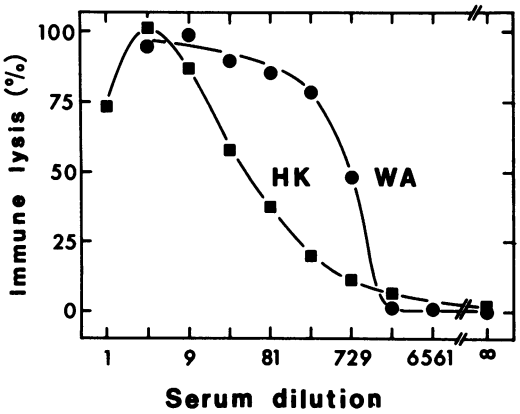

FIG. 3. Immune lysis of group A polysaccharide-coated vesicles for two different kinds of anti-A antiserum. HK is the human antiserum and WA is the Wellcome rabbit anti-A antiserum. The antigen (vesicle) concentration was nine times the normal one in this particular experiment.

The data for the HK and WA sera have been analyzed by statistical methods, and it was found that the microscopical dissociation constant (3) for the antibody-antigen reaction were identical for the two sera (A. I. Vistnes, submitted for publication). Differences in avidity seem therefore not to be the reason for the differences in shapes of the titration curves.

The observed cross-reactivity between the anti-C serum and the B liposomes in our assay is particularly interesting. Comparison of the structure of the two polysaccharides show only small differences. Both are composed of a linear homopolymer of sialic acid (*N*-acetylneuraminic acid) which for the group B polysaccharide is linked by α2—8 bonds and for group C by α2—9 bonds. In addition, there may be differences in the degree of O-acetylation. Although cross-reactions between B and C meningococcal polysaccharides are not observed by the common serological techniques, it is fully possible that our assay is more sensitive in this respect. If this cross-reactivity is real, it is also possible that the group C polysaccharide vaccine may have some protective effect against group B meningococci. Thus, the present experiments may offer an explanation for the slight increases of anti-B antibodies observed in four of five volunteers immunized with a mixed group C polysaccharide and type 2 protein vaccine (32).

Prozone effects (Fig. 2) are observed in many immunoassays, but they are not fully understood. The explanation may also be different in the different assays. The phenomenon has turned out to be very common with our present method, in contrast to our previous SMIA work (23). Suppression of bactericidal activity in low dilutions of immune sera is known as the Neisser-Wechsberg phenomenon (21a) and is commonly thought to be caused by an excess of antibody with a limited amount of complement. This may lead to the binding of antibodies to the bacterial cells, but for sterical reasons to an inadequate exposure of the complement-binding region of the antibody to the relatively large C1q molecule. Consequently, no lysis will occur. The same may be the situation with liposomes. We find that the prozone can be overcome either by reducing the amount of antibody or by increasing the amount of complement.

Inhibition of bactericidal activity by IgA antibodies in human meningococcal antisera has also been observed (13). The reason for this is that the IgA molecules bind to the antigens but do not activate the complement system. However, we do not think that this is the explanation here since a prozone effect was observed even for the monoclonal IgM antibodies.

Finally, it should also be mentioned that the various types of specific outer membrane proteins as well as lipopolysaccharides from *N. meningitidis* could probably be incorporated in the vesicle membrane in a way similar to the polysaccharides and allow the development of additional specific and sensitive serological assays. The potentials for the technique are therefore good. Presumably, it could be used as a general test for lytic antibodies in many host-parasite interactions.

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