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Outer-Membrane Protein and Lipopolysaccharide Serotyping of *Neisseria meningitidis* by Inhibition of a Solid-Phase Radioimmunoassay

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A new procedure involving inhibition of a solid-phase radioimmunoassay was developed for specific determination of the outer-membrane protein and the lipopolysaccharide (LPS) serotypes of meningococci. Antigen was allowed to bind to the wells of a polyvinyl microtiter plate and then reacted with a limiting amount of homologous antibody which had been preincubated with buffer or a standard concentration of inhibiting antigen. The amount of antibody bound per well was quantitated by incubation with excess ^{125}I-labeled goat anti-rabbit immunoglobulin. Typing sera for detecting eight LPS antigens and 18 protein antigens were made in rabbits by use of both the group C and group B bactericidal serotyping strains. Reactions between unabsorbed sera and purified LPS were inhibited in the LPS typing system, whereas reactions between absorbed sera and outer-membrane complex were inhibited in the protein typing system. Outer-membrane complex was used as the inhibiting antigen in both cases. Approximately 97% of the 80 group B and C strains tested were LPS typable, and 80% were protein typable. Of 51 group A strains tested, however, only 22% were LPS typable and 14% were protein typable. Several nonreciprocal correlations between the occurrence of particular LPS and protein serotype antigens on the same strain were observed, but in general the protein and LPS serotype antigens appeared to occur independently.

The subcapsular serotype antigens of *Neisseria meningitidis* have received increasing attention in recent years. These antigens, which are localized in the meningococcal outer membrane (6, 19, 20), are important in epidemiological studies (2, 8, 14), in studies on the mechanisms of natural immunity (10), and in studies directed toward the use of these antigens as vaccines against group B meningococcal disease. Although the serotype antigens have generally been considered to be outer-membrane proteins (6; F. A. Wyle and D. L. Kasper, Bacteriol. Proc., p. 99, 1971), we have recently demonstrated that meningococci may also be serotyped on the basis of their lipopolysaccharide (LPS) antigens (12). Several different serological procedures have been described for serotyping meningococci (3-5, 9, 12) and measuring antibodies to the serotype antigens (1, 7, 18, 20). Bactericidal serotyping (3, 9) and precipitin reactions in agar gels (4, 14) have been the most frequently used methods. These methods, however, do not clearly distinguish between the protein and LPS serotype antigens which are intimately associated in the outer membrane (6, 19, 20). Although selected serotype determinants have been correlated with the major outer-membrane protein (5, 6), anti-LPS antibodies are probably involved in some of the weaker bactericidal serotyping reactions (12). The existence of two different kinds of outer-membrane antigens that appear to have independent serotype specificities (12) suggests the need for serotyping and assay methods which are capable of clearly distinguishing between the two kinds of antigens.

In the present communication, we describe a solid-phase radioimmunoassay (SPRIA) inhibition method of serotyping that has allowed specific determination of both protein and LPS serotypes with a single antigen extract. The LPS serotyping system employed the same eight typing sera previously described for LPS serotyping by hemagglutination inhibition (12). The protein serotyping system, however, was made up of antisera to bactericidal serotyping strains characterized by Gold and Wyle (G-W strains; 8, 9) and Frasch and Chapman (F-C Strains; 3-5), as well as several additional strains characterized by the Department of Bacterial Diseases, Walter Reed Army Institute of Research (WR strains). By this system, the protein and LPS serotypes of 142 meningococcal strains, mostly of groups

A, B, and C, were determined, and the results were analyzed for possible correlations between the two serotypes.

MATERIALS AND METHODS

Bacterial strains and serotype nomenclature. The following group B strains of *N. meningitidis* were kindly supplied by Carl Frasch, Bureau of Biologics, Washington, D.C.: M1080, B16B6, M981, M992, M990, M986, M978, M982, M1011, M136, and S3032. Group A strains were kindly supplied by J. Sipple, Naval Medical Research Institute, Bethesda, Md., G. Filice, Center for Disease Control, Atlanta, Ga., and P. Helena Mäkelä, Central Public Health Laboratory, Helsinki, Finland. All other strains were from the culture collection of the Department of Bacterial Diseases, Walter Reed Army Institute of Research, Washington, D.C.

Media, growth conditions, and preservation of bacterial strains were as previously described (19).

The protein and LPS serotypes of meningococcal strains, as determined by the SPRIA inhibition method, are specified by using a "P" or "L" prefix, respectively. The antigenic profile of a strain is given in parentheses after the strain designation, using the format (serogroup:protein serotype:LPS serotype). For example, the antigenic profile of the group C strain 118V, which has protein antigens 3 and 4 and LPS antigens 2 and 4, is given as 118V(C:P3,4:L2,4).

Bactericidal serotypes are given using the original nomenclature of Gold and Wyle (9) or Frasch and Chapman (3), as appropriate.

Materials and reagents. Polystyrene "U" microtiter plates and polyvinyl, flexible "U" microtiter plates were obtained from Cooke Laboratory Products, Inc., Alexandria, Va.

The phosphate-buffered saline (PBS) was Dulbecco PBS (Grand Island Biological Co., Grand Island, N.Y.). PBS used to dilute antigen for coating the wells of the polyvinyl plates contained 0.02% phenol red as an aid in visualization. The "filter" diluent contained 10% (vol/vol) fetal calf serum, 0.2% sodium azide, and 0.02% phenol red in PBS (pH 7.4). Goat anti-rabbit immunoglobulin (GARG) was obtained from Antibodies Incorporated, Davis, Calif., and labeled with ^{125}I by the lactoperoxidase method as previously described (18). Subtilisin was obtained from Nutritional Biochemicals Co., Cleveland, Ohio, and Pronase, B grade, free of nucleases, was obtained from Calbiochem, La Jolla, Calif.

Preparation of antigens. Outer-membrane protein and capsular polysaccharide were prepared as previously described (20). LPS was prepared as previously described (20) or by the phenol-water method of Westphal et al. (17). Outer-membrane complex (OMC) was prepared from organisms grown either on BYE agar plates (Baltimore Biological Laboratory, Cockeysville, Md.) or in liquid medium as previously described (19). Normally the cells from four to six plates or from 100 to 200 ml of overnight liquid culture provided enough OMC for protein and LPS serotype determination as well as for analysis of the outer-membrane proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, if so desired. OMC was prepared as previously described (20). Briefly, the cells were suspended in about 25 ml of buffer containing 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride, 0.15 N NaCl, and 0.01 M ethylenediaminetetraacetic acid (pH 7.4). The suspension was heated at 60°C for 30 min, sheared in an Omnimixer (Dupont Instruments/Sorvall, Newtown, Conn.) at full speed for 2 to 3 min, and centrifuged at 20,000 × *g* for 15 min to remove cells and debris. OMC was then pelleted from the supernatant by centrifugation at 100,000 × *g* for 2 h. The pellet was washed by suspending in distilled water and repelleting as before. The final pellet was suspended in about 0.5 ml of distilled water, assayed for protein, and diluted with water to 1 mg of protein per ml. The standardized antigen preparations were stored at −20°C.

Preparation of antisera. Typing sera were prepared in 2-kg New Zealand white rabbits by giving three intravenous injections of 1×10^7, 1×10^8, and 5×10^8 live meningococci on days 0, 21, and 42, respectively. Rabbits were bled on day 52.

Absorption of antisera with bacterial cells was performed by mixing the serum with one-tenth to one-fifth volume of packed cells and placing the mixture on a rotator, first at 37°C for 1 h and then at 4°C for 1 h. The organisms were removed by centrifugation at 10,000 × *g* for 15 min, and the serum was filtered through a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.). Absorption of sera with LPS was performed by coating sheep erythrocytes with the LPS as previously described (20) and mixing 10 volumes of a 1:10 dilution of heat-inactivated serum with 1 volume of the packed erythrocytes. After 2 h at 37°C, the cells were removed by centrifugation at 2,000 × *g* for 10 min.

Digestion of OMC with proteolytic enzymes. OMC was diluted to 1 mg of protein per ml with PBS (pH 7.4) or first precipitated from 0.1 M NaCl with 4 volumes of ethanol and then centrifuged, and the pellet was suspended in PBS at 1 mg of protein per ml. Subtilisin or Pronase was added in the amount of 100 μg per mg of OMC protein, and sodium azide was added to 0.1% (wt/vol). Controls received PBS in place of enzyme. The tubes were capped and placed in a 37°C water bath overnight and then were placed in a boiling water bath for 4 min to inactivate the enzymes. Enzyme activity was assayed by using fluorescamine (Roche Diagnostics, Div. Hoffmann-La Roche, Inc., Nutley, N.J.) to measure the increase in primary amino groups with time (16).

Serotyping by SPRIA inhibition. The SPRIA inhibition was based on the quantitative SPRIA previously described (12), and the procedure and conditions were the same unless otherwise indicated. The test was performed in "U"-bottom microtiter plates and, in addition to the diluents described above, required four reagents: (i) LPS or OMC homologous to each typing serum was diluted in PBS to 25 or 100 μg/ml, respectively. (ii) The rabbit typing sera were diluted in filler to contain a limiting amount of specific antibody. The actual dilution was determined by performing the SPRIA as previously described (18) on serial dilutions of the typing serum. That dilution of serum which resulted in 20 to 30% of the maximum

or plateau level of ^{125}I bound was used with the same concentration of ^{125}I-labeled GARG for serotyping. The diluted serum contained approximately 0.1 μg of specific antibody protein per ml. (iii) The inhibitor or test antigen (usually OMC) was diluted in filler to 100 μg/ml, which was twice the concentration required to give maximum inhibition. (iv) ^{125}I-labeled GARG with a specific activity of about 5×10^5 cpm/μg was diluted in filler to give approximately 1.6 μg of specific antibody protein per ml. This was approximately 40 times the amount of primary antibody bound in the uninhibited control wells. Although the specific activity and concentration of ^{125}I-labeled GARG was not critical, it was important to use a sufficiently high concentration of ^{125}I-labeled GARG to saturate the binding sites on the type-specific antibody. Doubling the concentration of ^{125}I-labeled GARG should not result in more than a 10 to 20% increase in the cpm of ^{125}I bound.

A scheme of the test is given in Fig. 1. The wells of a flexible polyvinyl microtiter plate were coated with antigen by placing 25 μl of diluted antigen in the wells and allowing the antigen to bind for 1 h at 37°C. The unbound antigen was then aspirated, and the wells were rinsed with 50 μl of filler. Unoccupied binding sites were then filled by addition of 100 μl of filler and incubation at 37°C for 1 h. The solution was then dumped out, and the wells were washed twice with PBS. Inhibition mixtures consisting of 50 μl of diluted typing serum and 50 μl of test antigen (inhibitor) were placed in the wells of a separate polystyrene microtiter plate and allowed to react at 37°C for 1 h. Control wells contained filler only, filler plus typing serum, and homologous OMC plus typing serum. After incubation, duplicate 25-μl samples of the mixtures were placed in the antigen-coated wells of the flexible microtiter plate. Binding was allowed to proceed overnight at room temperature, after which the liquid was aspirated and the wells were washed once with filler and five times with PBS. ^{125}I-labeled GARG (25 μl) was then placed in each well except for two "no-inhibitor" control wells, which received double-strength ^{125}I-labeled GARG as a control to insure saturation binding to the type-specific antibody. The ^{125}I-labeled GARG was allowed to bind for at least 6 h (overnight if more convenient) at room temperature. The liquid was then aspirated, and the wells were washed once with filler and four times with PBS. The plates were held upside down, tapped on an absorbent towel to remove any remaining liquid, and allowed to dry. The wells were then cut off into tubes and counted.

Data processing. Calculations and tabulations of data were done with a programmable Hewlett-Packard model 9810 calculator with typewriter printout (Hewlett-Packard Co., Loveland, Colo.). Percent inhibition was calculated by using the following expression:

$$\text{percent inhibition} = 100 - \frac{\text{mean cpm bound with inhibitor} - \text{background}}{\text{mean cpm bound without inhibitor} - \text{background}} \times 100$$

Antigen-coated control wells that received filler in place of an inhibition mixture were used as the background controls.

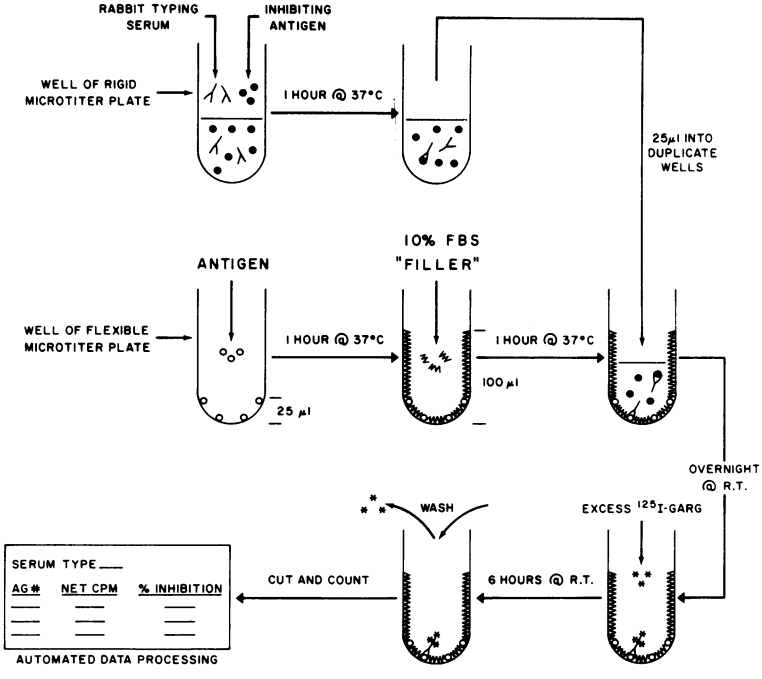

FIG. 1. *Scheme of the SPRIA inhibition method of serotyping. FBS, Fetal bovine serum; R.T., room temperature.*

RESULTS

Standardization and specificity of the serotyping reactions. Preliminary studies were performed to standardize the procedure and examine the specificity of the serotype reactions. The effect of the inhibiting antigen concentration on the level of inhibition was investigated to determine the concentration required to achieve maximum inhibition. The reaction between antiserum to strain 138I and the homologous OMC was inhibited by various concentrations of OMC from the homologous and several heterologous strains (Fig. 2). Maximum homologous and heterologous inhibition was achieved at a concentration of 50 μg of protein per ml. Inhibition by OMC from heterologous strains was approximately 20 to 30% at 100 μg/ml and presumably represents inhibition of antibody binding to common antigens. This level of background or nonspecific inhibition varied between 0 and 30 to 40% with different sera. Usually, nonspecific inhibition was in the range of 0 to 20%; if it was greater than 30%, the serum was not used for serotyping.

The specificity of the LPS serotype reactions for LPS determinants as opposed to protein serotype antigens was assured by the use of purified LPS as the homologous plate-bound antigen. The LPS typing sera and the corresponding immunizing strains were the same as previously described for LPS typing by hemagglutination inhibition (20). In a grid inhibition experiment with the eight LPS serotype reactions (Table 1), homologous OMC inhibited almost as well as the homologous, purified LPS controls. Five of the eight antigens inhibited greater than 30% of the binding in one or more of the heterologous reactions. These results indicate the presence of multiple LPS serotype determinants on these strains. Strains 6275 and 6155 (OMC numbers 3 and 7, respectively) both inhibited reactions L3 and L7 and are therefore both type L3,7. The specificities of the serotype reactions L3 and L7 may be closely related but do not appear to be identical, since some strains only inhibited one or the other of the reactions, e.g., M982(B:P9:L7) and 138I(C:P2:3).

Since the plate-bound antigen in the protein serotyping system was OMC (containing both protein and LPS serotype antigens), the specificity of the protein serotype reactions was

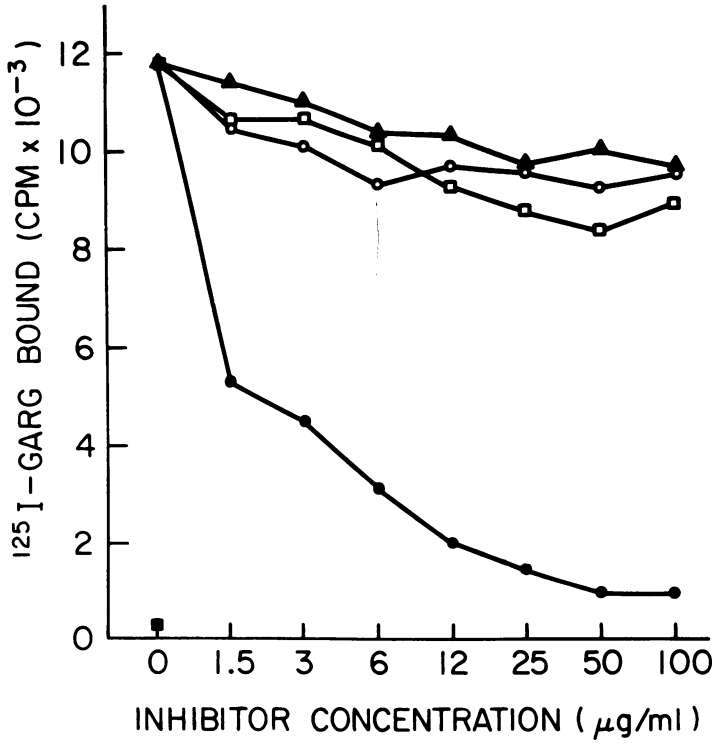

FIG. 2. *Effect of inhibitor (OMC) concentration on the amount of [^{125}I]GARG bound per well in the SPRIA inhibition serotyping reaction. The binding of rabbit anti-138I(C:P2:L3) to homologous plate-bound OMC was inhibited with 35E(C:P5:L2) OMC (▲), 126E(C:P3:L1,8) OMC (□), 118V(C:P3,4:L2,4) OMC (○), and 138I(C:P2:L3) OMC (●) at various concentrations.*

achieved by absorbing the typing sera, as needed, either with the homologous LPS to remove anti-LPS antibodies or with intact organisms of an appropriate heterologous strain to remove antiprotein antibodies with specificities other than those desired. Some of the typing sera were highly specific for a particular protein serotype antigen without absorption. The effectiveness of absorptions and the resulting specificity of the protein serotype reactions were examined by grid inhibition experiments in which the homologous LPS and capsular polysaccharide were included as inhibiting antigens (Table 2). The protein serotype reactions were very specific. Inhibition by the homologous LPS and group polysaccharide was in the range of 0 to 12%, whereas homologous inhibition by the OMC was 88 to 100%. Although OMC from several of the prototype strains inhibited heterologous reactions, heterologous inhibitions above 30% were relatively infrequent. Only inhibition greater than 30% is listed in Table 2, and inhibition of 50% or greater was considered positive for the purposes of serotyping.

Further evidence that the protein serotype reactions were specific for protein determinants was obtained by examining the effect of digestion with proteolytic enzymes on the ability of the OMC to inhibit the homologous serotype reaction. The inhibitor activity of OMC from eight of the prototype strains before and after digestion with subtilisin is given in Table 3. Inhibition of the protein serotype reactions, but not of the LPS serotype reactions, was decreased as a result of the subtilisin treatment. Because of the known resistance of bacterial outer-membrane proteins to digestion by proteolytic enzymes (15), the OMC was precipitated with 80% ethanol to partially remove phospholipids and increase exposure of the proteins to the enzymes.

TABLE 1. *Grid inhibition of LPS serotype reactions*

Serotype	Prototype strain	Percent inhibition by indicated OMC[a]								Homologous LPS
		1	2	3	4	5	6	7	8	
L1	126E(C)	98								98
L2	35E(C)		87							99
L3	6275(B)	60		95				56	53	99
L4	89I(C)				95				86	96
L5	M981(B)					94				99
L6	M992(B)						96			98
L7	6155(B)			59				90	63	99
L8	M978(B)	70							95	96

[a] OMC was derived from the strain listed opposite the serotype reaction of the corresponding number. Only inhibition that was greater than 30% is given.

TABLE 2. *Grid inhibition of protein serotype reactions*

Serotype reaction	Prototype strain	Percent inhibition by indicated OMC[a]																	Homologous	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	LPS	PS[b]
P1	60E(C)	98																		0	7
P2	M986(B)		94		36					33										0	0
P3	126E(C)			95	64															3	3
P4	118V(C)				93			73												0	0
P5	35E(C)					91														7	0
P6	32I(C)		73				97													0	0
P7	89I(C)							94												2	0
P8	190I(B)								97					51			44			0	0
P9	M982(B)									100										0	0
P10	M1011(B)										88									7	4
P11	M1080(B)											97								10	5
P12	S3032(B)												91							7	5
P13	6940(B)				35	31								94						0	0
P14	M981(B)														96		35			12	0
P15	M992(B)			33		43		32				39				90	32			7	4
P16	M990(B)																96			2	12
P17	6557(B)																	97		7	3
P18	M978(B)															33			92	0	0

[a] OMC was derived from the prototype strain listed opposite the serotype reaction with the indicated number.
[b] PS, Capsular polysaccharide.

The ethanol treatment alone had little effect on the inhibitory capacity of the OMC, but it did facilitate greater destruction of the protein serotype determinants by subtilisin. Inhibition of the LPS serotype reactions was unaffected by these treatments. Although the inhibitory capacity of the protein serotype determinants on the OMC of all eight strains was significantly decreased, in the case of 60E, 118V, and 35E the determinant remained partially resistant to digestion by proteolytic enzymes (Pronase was also tested with very similar results).

Description of the protein serotyping system. The protein serotyping system (Table 4) consisted of antisera to the G-W strains, the F-C strains, and four additional WR strains (3, 8). Absorptions were made as indicated in Table 4, and the sera were tested for specificity in grid inhibition experiments as described above. Several antisera made using the F-C strains were found to have specificities essentially identical to antisera made by using one of the G-W strains or WR strains. In these cases, the serum with the cleanest and most specific inhibition pattern was included in the typing system. The identity of the F-C type 2 antigen and the G-W type II

TABLE 3. *Effect of subtilisin digestion on inhibition of protein and LPS serotype reactions by OMC*

Serotype reaction[a]	Inhibiting OMC	Percent inhibition after indicated treatment				
		None	Control[b]	Subtilisin	Ethanol	Ethanol, then subtilisin
P1 (L3)	60E	90 (95)[c]	79 (88)	67 (89)	88 (95)	40 (94)
P2 (L3)	138I	93 (94)	90 (92)	38 (96)	86 (93)	18 (92)
P3 (L1)	126E	82 (98)	73 (94)	36 (95)	77 (98)	16 (98)
P4 (L2)	118V	96 (66)	96 (73)	74 (77)	86 (78)	63 (73)
P5 (L2)	35E	92 (59)	92 (71)	74 (77)	86 (68)	50 (64)
P6 (L2)	32I	95 (80)	95 (87)	7 (90)	94 (84)	0 (82)
P7 (L4)	89I	92 (86)	58 (77)	11 (93)	38 (75)	10 (82)
P10 (L3)	M1011	92 (93)	84 (89)	43 (92)	87 (94)	13 (95)

[a] Numbers with P prefix refer to protein serotype reactions, and those in parentheses refer to LPS serotype reactions.

[b] Control tubes received PBS in place of enzyme.

[c] Percent inhibition of indicated LPS serotype reaction is given in parentheses.

TABLE 4. *Description of SPRIA inhibition protein serotyping system*

SPRIA protein serotype	Immunizing strain	Absorbing strain or antigen	Characteristics of immunizing strains				
			Serogroup	Characterized by[a]:	Bactericidal serotype	SPRIA serotype	
						Protein	LPS
P1	60E		C	G-W	I	P1	L3,(4,7)[b]
P2	M986		B	F-C	2,7	P2,6	L3,(2,7)
P3	126E	LPS[c]	C	G-W	III	P3	L1,8
P4	118V	60E, LPS	C	G-W	IV,III	P4,3	L2,4
P5	35E	LPS	C	G-W	V	P5	L2
P6	32I	60E, 118V	C	G-W	VI,IV,I	P6	L2,4
P7	89I	118V	C	WR	VII,IV	P7,4	L4
P8	190I		B	WR	VIII	P8	L1,3,4,7
P9	M982		B	F-C	9	P9	L7
P10	M1011	B16B6	B	F-C	2,10	P10,6,2	L3,(7)
P11	M1080		B	F-C	1	P11	L3,4,7,8
P12	S3032		B	F-C	12	P12	L3,7
P13	6940	LPS	B	WR	XII,III	P13,(8)	L1
P14	M981	LPS	B	F-C	4	P14	L5
P15	M992	LPS	B	F-C	5	P15	L6
P16	M990	M136	B	F-C	6	P16	L3,(7)
P17	6557		B	WR	XI,III	P17	L1,8,(7)
P18	M978	M1080,[d] LPS	B	F-C	8,(1,3)	P18	L8,4,7,(3)

[a] G-W, Gold and Wyle (9); F-C, Frasch and Chapman (3); and WR, workers at Walter Reed Army Institute of Research.

[b] Parentheses indicate that inhibition was between 50 and 69%; in all other cases, inhibition was ≥70%.

[c] In each case, LPS was from the homologous strain.

[d] In this case, the organisms were treated with 0.1 N HCl for 2 h at 37°C and washed.

antigen has been demonstrated (11, 14). Thus, of antisera to six different type 2 (or II) strains, the antiserum to M986 (F-C type 2, 7) was chosen as the typing serum for type P2, because it was inhibited strongly (>80%) by OMC from all strains tested that were known to be type 2 (or II) by bactericidal serotyping. Another group of sera found to have a very similar pattern of inhibition included anti-M136 (F-C type 11) anti-89I (WR type IV, VII) and anti-118V (G-W type IV). In this case only antisera to strains 118V and 89I were included. Finally, anti-M982 (F-C type 9) was found to be very similar in specificity to anti-6155 (WR type X), which was omitted.

Suitably specific typing sera corresponding to the F-C types 3, 7, and 8 were not obtained prior to performing the serotyping reported in this paper. Antiserum to M978 (F-C type 8 [3, 1]) contained a relatively high level of antibodies to a common antigen. These antibodies were not easily removed by cross-absorption with viable organisms, but it was subsequently found that cross-absorption with heterologous organisms that had been treated with 0.1 N HCl for 2 h at 37°C was effective in removing the cross-reactive antibodies. After additional absorption with M978 LPS, this antiserum was found to have good specificity and is given in Table 3 as the typing serum for P18.

Extensive correlation between bactericidal serotyping and SPRIA inhibition serotyping has not been attempted, but generally good correlation between types P2 and P4 and the bactericidal types II(2) and IV(11), respectively, has been observed. In the case of several other types (e.g., P1, P3, P5), however, significant differences in the results of typing by the two methods have been observed.

Protein and LPS serotyping of 142 strains of meningococci. The SPRIA inhibition method of serotyping was used to investigate the distribution of protein and LPS serotype antigens among a cross section of 142 strains, mostly of groups A, B, and C, and to examine the correlation between the protein and LPS serotypes.

The distribution of 17 protein serotype antigens among the 142 strains is shown in Table 5. Only one of the 17 serotype antigens was found on the group A strains, which were isolated in eight different areas of the world. Eight of 51 strains, or 16%, were type P6, and the remaining 86% were nontypable. This suggests that group A strains do not share most of the major protein serotype antigens found on group B and C strains. Among the group B strains tested, the serotype antigens P2, P3, and P6 were most common. In many cases all three were found on the same strain, but they were also found to occur individually or with other protein serotype antigens. The distribution of protein serotype antigens among the group C strains was similar to that in group B strains except that P3, which was the most common group B antigen, was not found on any of the group C strains tested except the prototype strain. About 20% of the group B and C strains were nontypable. The antigens P1, P8, P12, P13, P14, P16, and P17 were not found on any of the 142 strains except the prototype strain and were therefore omitted from the table. Although only a few strains from serogroups other than A, B, and C were tested, 73% of them were typable.

The distribution of the 8 LPS serotype antigens among the same 142 strains is given in Table 6. Group A strains were again mostly nontypable (78%). All 11 of those that were typable had the L7 antigen(s) plus one or more of the antigens L4, L6, or L8. It thus appears that the LPS antigens of group A strains also differ significantly from those of group B and C strains and are not all identical. All non-group A strains except three were typable with the eight LPS typing sera. The results obtained here agreed with the results obtained by the hemagglutination inhibition method described previously (12).

The relationship between the occurrences of protein serotype antigens and LPS serotype an-

TABLE 5. *Occurrence of 17 protein serotypes among 142 meningococcal strains*

Serogroup	No. of strains	Protein serotype[a]										
		P2	P3	P4	P5	P6	P7	P9	P10	P11	P15	NT[b] (%)
A	51					8						43 (84)
B	55	17	20	5		14	1	2	9	2	3	12 (22)
C	25	13	1	4	1	11	1		7		2	4 (16)
Other[c]	11	3	1	1	2	6			1	1		3 (27)
Total	142	33	22	10	3	39	2	2	17	3	5	63 (44)

[a] Types P1, P8, P12, P13, P14, P16, and P18 were not detected except on the prototype strain. Many strains had multiple serotype antigens and thus appear under more than one serotype heading.

[b] NT, Nontypable.

[c] Seven from group Y and one each from groups X, Z, 29E, and W135.

TABLE 6. *Occurrence of 8 LPS serotypes among 142 meningococcal strains*

Serogroup	No. of strains	LPS serotype[a]								
		L1	L2	L3	L4	L5	L6	L7	L8	NT[b] (%)
A	51				7		8	11	4	40 (78)
B	55	8	19	30	17	8	5	37	6	1 (2)
C	25	2	6	17	9			7	3	1 (4)
Other[c]	11		5	5	1		1	7	1	1 (9)
Total	142	10	30	52	34	8	14	62	14	43 (30)

[a] Many strains had multiple serotype antigens and thus appear under more than one serotype heading.
[b] NT, Nontypable.
[c] Seven from group Y and one each from groups X, Z, 29E, and W135.

TABLE 7. *Correlation between the occurrence of protein and LPS serotype antigens*

Protein serotype[a]	No. of strains	No. with indicated LPS serotype[a]							
		L1	L2	L3	L4	L5	L6	L7	L8
P2	33	0	10	32	5	2	0	22	1
P3	22	3	11	11	7	6	1	11	2
P4	10	1	2	6	8	0	1	5	0
P6	39	1	7	24	4	1	0	15	1
P10	17	1	4	15	2	2	0	10	0

[a] Many strains have multiple serotype antigens and thus appear under more than one heading.

tigens on the meningococcal strains that were studied is shown in Table 7. The number of strains that had each of the more common protein serotype antigens is listed, along with the number of strains with a given protein serotype antigen having in addition each of the eight LPS serotype antigens. When looked at in this way, several relatively strong correlations are apparent. The P2 antigen was associated with the antigen L3 on 32 of 33 strains, P10 was associated with L3 on 15 of 17 strains, and P4 was associated with L4 on 8 of 10 strains. If the reverse is considered, however (i.e., how many strains with the type L3 antigen also have the type P2 antigen, or how many with the type L4 antigen also have the type P4 antigen?), the strong correlation does not hold up. The meaning of the correlations that are seen is not known, but overall the protein and LPS serotype antigens appear to behave as independent antigenic markers.

Multiple protein serotypes. The possibility that the presence of more than one protein serotype antigen associated with the same strain was the result of mixed cultures was investigated by plating a series of strains on solid medium and picking single colonies. OMC prepared from cultures grown from single colonies was then used for determination of the protein and LPS serotypes. These results (Table 8) indicate that a single strain may carry multiple protein and LPS serotype antigens.

TABLE 8. *Serotyping of strains grown from a single colony*

Strain	Serogroup	Protein serotype	LPS serotype
7641	B	P4,6	L3,4,(6,7)[a]
53I	B	P2,3,6	L3,7
6586	B	P3,(4)	L3,4
6556	Y	P4,10	L3,(7)
6298	B	P2,(3),6	L3,7

[a] Parentheses indicate inhibition between 50 and 69%.

DISCUSSION

Serotyping by SPRIA inhibition by using the combined set of 17 protein typing sera and 8 LPS typing sera results in a more complete antigenic description of group B and C meningococcal strains than was previously possible. This approach, however, also raises some questions regarding serotype nomenclature and the relationship between the serotype determined by SPRIA inhibition and that determined by bactericidal tests or other methods. Although the protein typing sera used in the present study were prepared by means of the same strains used by Gold and Wyle (8, 9) and by Frasch and Chapman (3, 4, 5) to prepare their bactericidal typing sera, the specificities expressed by these sera when used in the two different typing procedures may not always be identical. Possible involvement of anti-LPS antibodies in bactericidal serotyping (12), use of typing sera which are probably not monospecific for a single protein determinant, and basic differences in the functions required of the type-specific antibodies for expression in the two typing systems could all lead to differences in the results obtained. In those instances where strains had previously

been serotyped by the bactericidal method, we often found agreement between the SPRIA protein type and the bactericidal type (particularly in the case of P2 and P4), but some differences were also observed. For this reason, and the need to distinguish the protein type from the LPS type, we renumbered the different protein types and gave them a "P" prefix rather than attempt to use a combination of the two different bactericidal serotype designations. The LPS serotypes were similarly given an "L" prefix to distinguish them clearly from the protein serotypes. The more common "O" prefix for LPS serotype determinants was not used because it has not been established that these determinants are located on O-side chains. The antigenic profile of each strain was therefore expressed using the format (serogroup:protein serotype:LPS serotype).

Experiments designed to demonstrate that the antigens involved in the protein serotyping reactions are indeed protein were not conclusive in the case of types P1, P4, and P5. A significant amount of inhibitory activity persisted after treatment of the OMC with proteolytic enzymes. Partial resistance of these membrane antigens to attack by proteolytic enzymes is not surprising, however, since proteins in the gram-negative outer membrane are known to be relatively resistant to attack by proteolytic enzymes (15). Since a significant decrease in antigenic activity was observed after subtilisin treatment, and partial removal of lipid by ethanol precipitation resulted in more extensive destruction of antigenic activity, it seems probable that the partial resistance of these antigens to proteolytic enzymes was due to their inaccessibility to the enzymes rather than to a nonprotein composition. This conclusion is supported by the fact that no decrease at all in the capacity of the LPS component of the OMC to inhibit the appropriate LPS serotype reaction was observed after treatment with proteolytic enzymes.

Even though the typing sera were generally found to have a high degree of specificity for a particular serotype antigen, it is not likely that the sera are monospecific. Many of these serotype reactions were inhibited at a low level (0 to 30%) by OMC from most other strains tested. This suggests the presence of antibodies to common or cross-reactive determinants. The presence of multiple protein and LPS serotype determinants on many strains also suggests that the serotype specificity defined by some of the serotype reactions may involve more than a single determinant. The present method of serotyping, however, should allow precise definition of each specificity based on quantitative results of inhibition by an appropriate set of test antigens.

When the specificities of the combined set of protein typing sera were compared by grid inhibition experiments, surprisingly little duplication was observed. Only three serotypes (P2, P4, and P9) were detected by antisera to both a G-W or WR strain and an F-C strain. In these cases the serum with the poorest specificity was deleted from the set of typing sera. The resulting 18 typing sera all had different specificities. One advantage in using the combined set of typing sera is evident from the observation that several of the most commonly occurring serotypes (P3, P6, and P10) would not have been detected if typing sera to only the F-C strains or the G-W strains had been used. Even with the combined set of 18 typing sera, only about 78% of the group B and C strains were typable. On the other hand, about half of the serotypes were rarely detected or not detected at all except on the prototype strain. These results suggest the existence of a relatively large number of different protein serotype determinants among group B and C meningococci. Since a higher percentage of strains were typable by a much smaller number of LPS typing sera, LPS serotyping may be more useful than protein serotyping in some epidemiological studies.

Among 51 group A strains tested, 78% were nontypable in the LPS system and 86% were nontypable in the protein system. Only 4 of the 8 LPS types and 1 of 17 protein types were detected among the group A strains. These results indicate that many of the protein and LPS serotype determinants that are common to both group B and group C strains (12, 14) are less common or nonexistent among group A strains. Differences among group A strains, however, were demonstrated by both protein and LPS serotyping. Typing sera prepared with group A strains have recently been used successfully to determine both the protein and LPS serotypes of over 80% of the group A strains tested by SPRIA inhibition (manuscript in preparation).

The significance of several nonreciprocal correlations that were observed between the occurrence of certain protein and LPS serotype antigens (e.g., P2 and L3 or P4 and L4) on the same strains is not yet clear. Epidemiological studies involving a larger number of strains will be required to examine this question and to determine whether particular LPS serotypes have epidemiological significance such as that ascribed to the protein serotype 2 (2, 8, 14). Overall, the protein and LPS serotype antigens appear to occur independently, and therefore the serotype of each must be separately determined to obtain

an accurate antigenic profile of meningococcal strains.

The presence of multiple protein and LPS serotype determinants (up to four or five) on a single strain results in a relatively complex antigenic profile. In light of reports of significant phenotypic variation in outer-membrane components as a result of differences in growth conditions (7, 13), it will be important to determine to what extent the results of serotyping by SPRIA inhibition are affected by such variations. The protein serotype antigens P3, P6, and P10 often occur together with P2, which has been reported to be located on the major outer-membrane protein (6). The question arises of whether the serotype antigens P3, P6, and P10, which are all susceptible to degradation by proteolytic enzymes and therefore protein in nature, are also located on the major outer-membrane protein.

The SPRIA inhibition method of serotyping has certain significant advantages over existing methods of serotyping. Among these advantages are (i) separate, specific determination of the protein and LPS serotypes using a single antigen preparation, (ii) no requirement for complement, and (iii) quantitative results which are easily subjected to automated data reduction. Modification of the method by substitution of enzyme-linked GARG in place of the ^{125}I-labeled GARG would allow the present method to be used in laboratories where gamma-counting equipment is not available. In addition, we have found the SPRIA inhibition procedure to be generally useful for studies of antigenic specificity involving a variety of protein and membrane-associated antigens.

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