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Relationship of Serogroups of *Neisseria meningitidis*

II. R Variants of *Neisseria meningitidis*

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Meningococci isolated in primary cultures from nasopharyngeal carriers occasionally consisted of mixtures of smooth (S) and rough (R) strains. The R strains were separated from the S strains and their morphological and serological characteristics were studied. Some of these R strains reverted spontaneously to S strains which subsequently produced group-specific polysaccharide. Several R strains, grown in the presence of deoxyribonucleic acid from either an R strain of known parentage or an S strain, formed recombinants with serological group specificity.

Rough (R) and smooth (S) colonies of meningococci were mentioned by Branham (6) and were studied by Enders (9) and Maegraith (17) in 1932 and 1933, respectively. S and R colonies on blood-agar plates were described more completely by Rake (21). Alexander and Redman (2) found S colonies on Mueller-Hinton agar to be iridescent when illuminated by transmitted light, but R colonies lacked this property. These authors transformed S and R colonies of group C meningococci with group A deoxyribonucleic acid (DNA) to meningococci with group A specificity. They also raised the question of whether nongroupable meningococci of the nasopharynx might be transformed in vitro or in vivo to S virulent groupable meningococci. Furthermore, these authors reported that they were "investigating the susceptibility of nonclassifiable strains to in vitro inductions of type-specific traits." However, to our knowledge, no further report on this investigation has appeared in the literature.

Jackson, MacLeod, and Krauss (11) recovered S encapsulated VIII pneumococci from reciprocal transformations between two rough nonencapsulated mutants of S encapsulated VIII parentage. DNA extracts of R VIII strains also transformed R II strains to S VIII and S II strains. S recombinants were obtained from transformations of R II strains by DNA extracts of several S encapsulated strains. These recombinants were found to produce the type-specific polysaccharide of either the donor or the parent of the recipient.

Catlin (7) transformed antibiotic-sensitive meningococcal cells to antibiotic-resistant cells by using DNA and culture slime from meningococcal cells having antibiotic-resistant markers. She also suggested that transformation may take place in the nasopharynx and create "new genetic combinations with a greater range of adaptability."

Devine and Hagerman (8) noted the frequent occurrence of nongroupable strains of meningococci isolated from nasopharyngeal (NP) cultures of carriers and reported on the serological relationship of the RAS-10 strain to other serological groups. This strain was considered to be a strain lacking group-specific polysaccharide. The results of further investigation of the colonial morphology and serological relationships of nongroupable and groupable meningococcal strains are the subject of this report.

The designations R and S in this paper are used to distinguish between two categories of meningococci. Wilson and Miles (23) described six properties which distinguish R from S strains for bacteria in general. We studied three of these properties which we found to be applicable to meningococci. These three properties of the R forms of meningococci which differ from the S forms of meningococci as described by Wilson and Miles are: (i) loss of the antigenic components characterizing the surface of the cell in the S form, whether or not this component is present as a capsule; (ii) loss of virulence, partial or complete; (iii) change in colony form usually, but not always, in the direction of increased granularity and roughness.

MATERIALS AND METHODS

Strains. Strain M60 of *Neisseria meningitidis* was obtained from Margaret Pittman of the Bureau of Biologics Standards, National Institutes of Health

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(NIH). NIH strain M158 was obtained from ATCC as strain 13113. The RAS-10 strain was previously described (8). The identification and sources of the remaining S and R meningococcal strains used in this investigation are shown in Tables 1–3.

Maintenance of strains. Meningococcal strains were inoculated onto Mueller-Hinton agar (MHA, BBL) and grown in an 8% CO_2 atmosphere at 37 C for 16 to 18 hr. Several 2-mm loopsful of these cells were placed in 2 ml of Mueller-Hinton broth (MHB, BBL) in 5-ml screw-capped vials, suspended evenly with a Vortex mixer and frozen and stored at −70 C. Strains were recovered from storage by using a pipette to scrape about 0.05 ml of inocula from the surface of the frozen samples. The vials were returned immediately to storage at −70 C. This procedure conserved time, space, and materials since the same samples were utilized repeatedly as a source for these cultures.

Identification of S and R strains. The strains were inoculated on the surface of 15 ml of MHA contained in 100 by 15 mm plastic petri dishes to obtain well isolated colonies. After incubation for 16 to 20 hr at 37 C in 8% CO_2 atmosphere with 80% humidity, the colonies were observed using a model BVB-73 Stereo Zoom binocular microscope (Bausch & Lomb) with a 10× magnification. Illumination was provided by a Spencer illuminating lamp with a blue filter directed at an oblique angle to the mirror.

Slide agglutination test. Slide agglutination tests were performed as previously described (8).

Preparation of antigens. Meningococcal strains were inoculated on MHA to obtain confluent growth in 100 by 15 mm petri dishes and were incubated for 16 to 18 hr as for preparation of cultures for storage. One milliliter of 0.15 M NaCl was added to each dish; the cells were suspended in the liquid and transferred to 50-ml test tubes. Ten milliliters of 0.15 M NaCl was added to the test tube, suspended with a Vortex mixer, and diluted to match McFarland standards as explained below.

Antibody production. New Zealand white rabbits weighing approximately 2 kg were used for production of antibody. They were injected in the marginal ear vein with 0.25-, 0.25-, and 0.5-ml samples of suspensions of meningococci in 0.15 M NaCl containing 0.5% Formalin on days 1, 2, and 3, respectively. The concentration of the cells corresponded to a no. 3 McFarland standard. One week after the initial injection, 0.5-, 0.5-, and 1-ml samples of an identical suspension of live cells in 0.15 M NaCl were injected on three successive days, respectively. One week after the first injection of the second series, each rabbit was injected with a live suspension of cells in 1.0-, 1.0-, and 2-ml samples corresponding to no. 10 McFarland Standard on 3 consecutive days, respectively. One week later, the rabbits were bled by intracardial puncture, and antisera were separated and stored at −70 C.

Preparation of antibiotic-resistant strains. A heavy inoculum consisting of three 2-mm loopsful of meningococci taken from an 18-hr culture on MHA was spread evenly on each of three MHA plates containing screening concentrations of the antibiotics. To isolate pure cultures of antibiotic-resistant strains, resistant colonies appearing in 24 to 48 hr were subcultured on plates containing antibiotics. The screening concentrations of antibiotics were (μg/ml): streptomycin, 100; rifampin, 2; nalidixic acid, 10; and coumermycin A_1, 0.02. We had no antibiotic marker that appeared to be linked to serogroup loci but the yield of serogroup recombinants, although not measured, was increased by incubation for 24 hr.

DNA preparation. DNA was prepared by lysing the cells in 1% sodium dodecyl sulfate and precipitating DNA with ethanol as described by Marmur (18) as modified by Bövre (5) and Lie (15). DNA preparations obtained by this method contained more than 200 μg of DNA/ml.

DNA transformation. Procedures similar to those of Lie (15), Catlin (7), and Alexander and Redman (2) were utilized. A 0.1-ml sample of the DNA solution (200 μg of DNA/ml) was added to 1 ml of an MHB culture of meningococci containing approximately 10^6 cells in the log growth phase and incubated at 37 C for 24 hr. Deoxyribonuclease was not used to interrupt DNA absorption. A 0.1-ml sample of the culture was spread evenly on the surface of MHA plates containing appropriate concentrations of antibiotics for the selection of antibiotic-resistant transformants. When antibiotics were not used in the selection of transformants, a 5-mm loopful of the surface growth of the MHB culture was spread on MHA plates to obtain isolated colonies. Control cultures with no DNA added were handled as above to detect the occurrence of spontaneous reversion to S forms. Some DNA transformations were performed by using culture slime or by growing mixed cultures by the method of Catlin (7).

Mouse virulence. R forms of meningococci were tested for mouse virulence by the methods of Miller and Castles (19).

RESULTS

Colonial morphology of S meningococci. S meningococcal colonies of serological groups A, B, C, X, Y, and Z′ vary in appearance when observed on MHA as described above. Typical morphology varied to some degree between lots of MHA; however, colonies always appeared most typical when well isolated and between 16- and 20-hr old. These colonies were highly iridescent, transparent to translucent, blue with yellow tinge, and nearly agranular. After 20 to 48 hr of incubation, these S colonies became opaque, granular, and morphologically indistinguishable from the R forms described below. A series of colonial variants were observed with increasing degrees of transition from blue to white. Highly granular opaque white colonies were characteristic of colonies at the extreme end of this series. Frequently, colonies could be isolated and pure cultures maintained with stable characteristic morphological features. When the white opaque colonies were observed, either in a

wet mount or stained with the Gram stain, the maiority of the meningococcal cells were autolyzed and had lost their morphological integrity. In contrast, when the cells of iridescent or smooth blue colonies were similarly observed, they appeared morphologically as typical diplococci. Opacity appeared to be positively correlated with the degree of autolysis.

Colonial morphology of R strains. R strains of meningococci formed colonies which differed from the colonies of the S strain in that the former lacked the highly iridescent or blue appearance. Colonial morphological variants of the R strains were of increasing granularity and opacity and corresponded to the opaque variants of the S strains. The more granular colonial variants of the S and R strains were morphologically indistinguishable from each other, but were serologically identifiable by slide agglutination and gel diffusion tests.

Recognition of R strains by slide agglutination. Twenty-three per cent of the strains isolated from the nasopharynges of recruits arriving at Recruit Training Command, Great Lakes, since June of 1967 were nongroupable meningococci. The majority of these nongroupable strains were agglutinated with RAS-10 or RAS′ antisera (8).

In addition to the RAS-10 strain, two other R strains, RAS′469 and RC408, were found in pure culture from primary isolations of NP carriers at Greak Lakes, and a third strain, Flor351, was isolated from the spinal fluid of a patient with clinical meningococcal disease. This case occurred during an epidemic in which meningococci and echovirus 9 were implicated as the etiological agents (14). These strains differed serologically from each other, as well as from the RAS-10 strain. The R strain, Flor351, had been passed several times before we obtained the culture and may originally have been an S strain.

Nearly all of the nongroupable strains isolated from carriers were identified by either the RAS-10 or one of the three other antisera by the slide agglutination test. Antisera to one of these strains usually agglutinated a strain more strongly than any of the other R antisera; however, most of these strains cross-reacted to some degree. These four R antisera were used to categorize the R strains by slide agglutination tests as shown in Tables 1 and 2. Additionally, antisera were prepared to strains which were weakly agglutinated by these four antisera: $RB235_2$, RY14, and RX13. These three additional antisera strongly agglutinated only their homologous cells.

Based on the morphological and serological observations described above, a collection of R and S strains were accumulated and used in this study. A list of these strains and the sources thereof is shown in Tables 1 and 2.

Pathogenicity of R strains. In our experience, the only R strains that may have been the responsible etiological agent for meningococcal disease were the Flor351 strain mentioned above (Table 1) and a RAS-10 strain isolated from the conjunctiva of a patient with purulent meningitis at USNH, Great Lakes. We have attempted to demonstrate pathogenicity of numerous R strains for mice but have always been unsuccessful.

Identification of group-specific polysaccharide serogroup genotype of R strains isolated from NP cultures of meningococcal carriers. R and S strains of meningococci were separated from primary mixed NP cultures of 14 meningococcal carriers at Great Lakes. The S strains accompany-

TABLE 1. *Sources of additional smooth (S) and rough (R) strains with identification of the antisera most strongly agglutinating the R strain and method by which the S parentage of the R strain was determined*

R strain	Antisera agglutinating the R strain	Source of R strain	Method of identification of parent group of R strain	S parentage of R strain
RY10 (RAS-10)	RAS-10	NP[a] pure culture	—[b]	Y
RC408	RC408	NP pure culture	—[b]	C
RY295	RAS-10	NP pure culture	—[b]	Y
RAS′469	RAS′469	NP pure culture	NI[c]	NI
Flor351	Flor351	SF[d]	NI	NI
RA MK815[e]	RX13	Nitrous acid treatment	—[a,f]	A

[a] Nasopharyngeal.
[b] Allogenic transformation to parent serological group.
[c] Not identified.
[d] Recieved from L. Levitt, Manatee County Health Department, Bradenton, Fla.
[e] S form A MK815 received from W. Sanborn, Naval Medical Research Unit No. 3 (NAMRU-3), Cairo, United Arab Republic, as A MK815 and mutated to R form by nitrous acid.
[f] Mutagenic treatment with nitrous acid of pure culture of known serological group.

TABLE 2. *Identification of rough (R) variants in primary mixed nasopharyngeal cultures of meningococcal carriers at Great Lakes*

Smooth strain	R variant	Method of identification of parent group of R variant	Parent serogroup of R variant	Antisera agglutinating R variant
B724	RB724	—[a]	B	Flor351
$B235_1$	$RB235_2$[b]	—[a]	B	$RB235_2$
C198	RC198	—[c]	C	RAS-10
C468	RY468	—[c]	Y	RAS-10
C581	RC581	—[c]	C	RAS-10
C730	RY730	—[a]	Y	RAS-10
C407	RC407	—[a]	C	RAS-10
C372	RC372	—[a]	C	RAS-10
C599	R599	NI[d]	NI[d]	RAS-10
X13	RX13	—[c]	X	RX13
Y130	RY130	—[c]	Y	RAS-10
Y42	RY42	—[c]	Y	RAS-10
Y14	RY14	—[a]	Y	RY14
Z'94	RZ'94	—[a]	Z'	RAS'469

[a] Allogenic transformation to parent serological group.
[b] A nonreverting R strain of $B235_1$ was isolated by consecutive serial passage of $RB235_1$ and was designated $RB235_2$.
[c] Spontaneous reversion to parent serological group.
[d] Not identified.

ing the R strains included two group B, seven group C, three group Y, and one each of X and Z'. Seven R strains isolated from the 14 mixed cultures spontaneously reverted to S strains as noted in Table 2. Allogenic transformants were obtained from six of the seven remaining R strains. Five of these transformants were serologically identical to the S strain associated with the R strain in the primary mixed NP cultures. A total of 3 of 14 strains did not return to the serological group of the S strains found in the primary mixed culture. RY468 and RY730 were isolated with an S strain of C. The former strain spontaneously reverted to a group Y strain and the latter was transformed with DNA of $RB235_2$ to a group Y strain. These results imply that these individuals carried S forms mixed with R forms of another serogroup. Only 1 of 14 R strains (R599) was not identified, although it was isolated with a group C strain.

Additional R strains were identified as shown in Table 1. Strains RAS'469 and Flor351 were not identified since only S transformants of the donor strain were obtained from the former and the latter strain appeared to be incompetent.

Spontaneous R < − > S mutations. Despite several successive serial passages of individual colonies, 7 of 14 R strains isolated from primary cultures mixed with S strains and listed in Table 2 spontaneously mutated to S strains. The results suggest that two of these seven strains also mutated in vivo. An R strain was isolated from the nasopharynx of a carrier, and, on the following day, an S strain (Y42) was isolated in pure culture from the nasopharynx of the same man. When a single colony of this strain was passed on MHA, an R strain (RY42) was isolated from an opaque wedge which appeared in an iridescent blue colony. In another instance, a group Y strain was isolated from an NP carrier on four successive cultures, but, when the fifth culture was taken, an R strain was isolated. When the R strain was transferred daily on MHA, S strains of group Y (Y130) were isolated in each passage either from single colonies or from iridescent blue wedges of R (RY130) colonies.

An example of a spontaneously reverting R colony is shown in Fig. 1. Among several hundred opaque R colonies of strain RC581 on a plate, this colony developed a highly iridescent blue wedge. Colonies from a subculture of this wedge were group C meningococci. S revertants of $RB235_1$, RC581, and RC198 were recognized only after incubation for 1 to 3 days on MHA and were isolated in pure culture as in the preceding example.

Attempts to obtain S revertants of RY468 and RX13 by consecutive daily plating on MHA were unsuccessful. However, by using the surface growth of 1-day-old MHB cultures as an inoculum on MHA, S revertants were obtained.

Nonreverting R forms from some strains were difficult to isolate. A stable nonreverting R strain was sought from the unstable strain $RB235_1$, and colonies of each of 30 successive serial

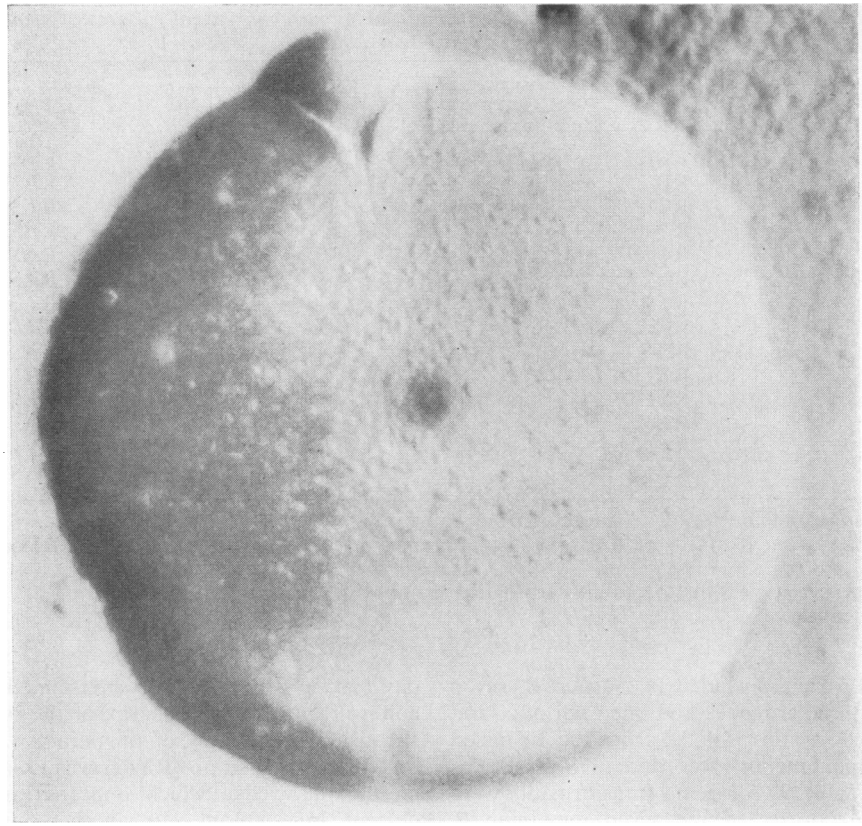

FIG. 1. *This colony of CR581 shows a pie-shaped wedge which is transparent blue and highly iridescent. This wedge appeared after incubation for 2 days, and a subculture of the wedge yielded a pure culture of group C meningococci.*

passages were examined without success. On the 30th passage, an S mutant of $RB235_1$ was passed on MHA and three R mutants were selected and passed. One of these three R mutants was stable on passage and was designated $RB235_2$.

Transformations. R strains of known parentage, RAMK815 and $RB235_2$, were transformed by DNA of S strains of serological groups B and C, respectively, and S forms of the donor and parent serogroup of the R strains were isolated in each instance.

Transformation experiments with the RAS-10 strain as the recipient were conducted by using DNA of several R strains and yielded S strains of group Y. Transformation of RAS-10 with DNA of S strains of group B yielded S serogroup transformants of either B or Y, or both, and DNA of S strains of C yielded S transformants of serological groups C or Y, or a mixture of both serological groups. The S strains obtained by recombination were all stable through many serial passages. The results of transformation of RAS-10 are shown in Table 3.

Transformation of two group D strains (ATCC 13113 and M60) was not successful, since they were either incompetent or of low competence. However, DNA from these D strains transformed two R strains ($RB235_2$ and RB724) to either group C or a mixture of groups B and C (*unpublished data*).

DISCUSSION

R and S forms and colonial variants of many other genera and species of bacteria have been studied extensively (1, 11–13). R forms of *Salmonella* played an important role in serological, biochemical, and genetic studies (4, 16, 20, 23). Although morphologically rough colonies and R forms of meningococci have been described, they have attracted little interest. Since Slaterus (22) described groups X, Y, Z, and Z′ in 1961 and Evans, Artenstein, and Hunter described 135 in 1968 (10), many strains formerly desig-

TABLE 3. *Serogroup transformation of RAS-10 540 recipients by DNA of smooth (S) and rough (R) donor strains*

Donor strain	Method of transformation	S recombinants isolated
RC581 (rR)[a]	DNA	Y (rR)[b]
$RB235_2$ (rR)	DNA	Y (rR)[b]
Flor351	Mixed culture	Y[c]
RAS'469	Mixed culture	Y[c]
RASF1[d]	DNA	Y[c]
B42[d] (rR)	DNA	Y (rR) + B (rR)[b]
B429 (rStrI)[e]	DNA	Y (rStrI)[b]
C930[d] (rStrI)	DNA	Y (rStrI)[b] + C (sStrI)[b]

[a] Rifampin resistant.
[b] Marker selected.
[c] Identified by colonial morphology and confirmed by agglutination by group-specific antiserum.
[d] Isolated from nasopharynx of carriers at Great Lakes.
[e] Resistant streptomycin independent.

nated as "nontypable" can be identified as legitimate serological groups. However, a high percentage of strains isolated from NP carriers at Great Lakes were not specifically agglutinated by antisera to any of the known serological groups: A, B, C, X, Y, Z, Z', and 135. These strains did show a fine granular agglutination in slide agglutination tests with homologous antisera as opposed to the large flocculation observed with groupable meningococci in their homologous antisera. Seven of the "nongroupable" strains listed in Table 2 were unequivocally R strains, since they spontaneously reverted to meningococci that agglutinated with group-specific antisera.

To determine the genotype with respect to group-specific polysaccharide antigens of stable nonreverting R strains, DNA transformations were performed to produce recombinants of these strains with R or S strains of known genotype. The first strain examined by this method was RAS-10 540. The S recombinants isolated, when strain RAS-10 540 was used as the recipient, grouped as either Y or the group of the donor. These results indicated that strain RAS-10 540 was an R form of group Y and were analogous to the results obtained in similar experiments with S and R pneumococci by Jackson et al. (11). Two R forms of known genotype, $RB235_2$ and RA MK815, were transformed to both their parent serogroup and the serogroup of the donor.

Fourteen R strains were separated from primary mixed NP cultures of 14 meningococcal carriers at Great Lakes. Each of these cultures was a mixture of an R strain with a serologically identifiable S strain. The serogroup of the parent of 13 of the 14 R strains was determined by spontaneous reversion or by DNA transformation. The genotype of the serogroup of 11 of these 14 R strains was the same as the S strains with which they were found in the original NP culture. Three exceptions were a strain R599, which was not identified, and two RY strains, which were isolated with S strains of group C. It should be noted that group Y was the most commonly found serological group in this population (3).

These 14 R strains, isolated from the NP of carriers in mixed cultures on primary plates, were morphologically and serologically identical to one or another of R strains frequently isolated in pure cultures from NP carriers. It should be added that the above mixtures of R and S colonies from primary cultures of the nasopharynges are found infrequently and most primary NP cultures were pure cultures of either S or R strains However, strains isolated from the same individual on successive days may occasionally alternate between a pure culture of an S strain and a pure culture of an R strain.

Nearly all publications on the epidemiology of the meningococci refer to nongroupable strains of meningococci. These strains are R forms of the S forms isolated in the population. The percentage of these R forms is probably distributed with a frequency proportional to the frequency of the distribution of the S forms isolated from the people in the population.

In a previous study (*unpublished data*), we tried to encourage the spread of R strains in a recruit population by selective treatment of group C carriers with minocycline and rifampin. The failure of this approach was probably due to many factors, one of which may have been the relative genetic instability of the R forms. Many of the R strains probably reverted to encapsulated S strains of group C and subsequently reinfected the population.

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