Association of Intrastrain Phase Variation in Quantity of Capsular Polysaccharide and Teichoic Acid with the Virulence of *Streptococcus pneumoniae*

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The pneumococcus undergoes spontaneous phase variation between an opaque and a transparent colony form. In an animal model of systemic infection following intraperitoneal inoculation of mice, the opaque phenotype was significantly more virulent than the transparent for each of 3 strains examined. The opaque phenotype was associated with 1.2- to 5.6-fold greater amounts of capsular polysaccharide compared with the transparent using a sandwich ELISA. A similar technique comparing the amount of total teichoic acid showed that the transparent phenotype had 2.1- to 3.8-fold more immunodetectable teichoic acid. This difference was confirmed by comparing the incorporation of [^{3}H]choline into teichoic acid. Cell fractionation revealed that variation in quantity of incorporated choline was due to differences in cell wall–associated teichoic acid. Results suggest that the pneumococcus phase varies between a virulent form with more capsular polysaccharide and less teichoic acid and an avirulent form with less capsular polysaccharide and more teichoic acid.

Streptococcus pneumoniae, the pneumococcus, colonizes the human nasopharynx and is a leading etiologic agent of upper and lower respiratory tract infection. In addition to causing disease primarily confined to the mucosal surface of the respiratory tract, the pneumococcus commonly causes bacteremia and infections that result from dissemination via the bloodstream. Characteristics of the organism that allow it to colonize the mucosal surface of the nasopharynx and to survive within the bloodstream are not completely understood.

This laboratory has described phenotypic variation in the pneumococcus [1]. These phenotypes are distinguished by their colony morphology when viewed with oblique transmitted light on transparent agar surfaces. There is spontaneous back-and-forth switching or phase variation of opaque, transparent, and in some strains intermediate forms that vary from isolate to isolate at a rate of 10^{-3}–10^{-6} per generation. Phenotypic variation in opacity is associated with differences in multiple surface determinants that may contribute to the ability of the organism to interact with its host.

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Organisms with the transparent phenotype have been shown to be more efficient than opaque organisms of the same strain at colonization of the nasopharynx in an infant rat model of carriage. The ability of transparent pneumococci to reside in their niche in the nasopharynx correlates with greater adherence to human lung epithelial cells (type II pneumocytes) [2]. Adherence to cells is augmented by cytokine stimulation with either tumor necrosis factor-β or interleukin-1 and appears to be mediated by interaction with the receptor for platelet activating factor (rPAF) [3]. In addition, it has been reported that the pneumococcus binds to the asialo-GM1 glycolipid [4, 5]. Both of these receptor activities require the expression of phosphorylcholine (ChoP) on the bacterial cell surface. The natural ligand of rPAF also contains ChoP, suggesting that the pneumococcus may be mimicking this structure to utilize its receptor.

The pneumococcus obtains choline from the growth medium and incorporates it in the form of ChoP into teichoic acids. In the pneumococcus both the cell wall–associated teichoic acid (C-polysaccharide) and the membrane-attached lipoteichoic acid (Forssman antigen) share a similar repeating structure [6]. Choline, a component of host membrane lipids, is found in few prokaryotes but recently has been shown to be a component of the surface glycolipids of two other major pathogens residing in the human respiratory tract, *Haemophilus influenzae* and *Mycoplasma* species [7, 8]. In addition to interacting directly with host receptors, ChoP in the pneumococcus also functions as an anchor for surface proteins referred to as choline-binding proteins. These proteins include the major autolysin (LytA), which is present at higher levels in the more autolytic transparent variants, and the abundant, antigenically variable pneumococcal surface protein A (PspA), whose function is unknown [9, 10]. The recently identified choline-binding protein CbpA is also present in higher levels in transparent compared with

opaque organisms and is required for efficient colonization in the infant rat model [11].

The critical contribution of the antiphagocytic capsular polysaccharide to the ability of the pneumococcus to cause invasive infection is well established [12]. The role of the capsular polysaccharide in virulence is readily demonstrated in mouse models of sepsis, although only certain types are highly virulent in mice [13]. *S. pneumoniae* is capable of expressing 90 distinct polysaccharides that form the basis for typing. In addition to exhibiting this remarkable diversity in capsule types, this organism shows variation among isolates of the same type in amounts of capsular polysaccharide produced [14]. There is a correlation between increased amounts of capsular polysaccharide and greater virulence in mice [14]. The relative contribution of other putative virulence factors, including a thiol-activated toxin, pneumolysin, and PspA to pneumococcal infection, is less clear-cut [15, 16]

This study examines the effect of differences in colony morphology on invasive pneumococcal infection. Results show that, in contrast to the events that occur during colonization, which select organisms of the transparent phenotype, during systemic infection, opaque organisms are more virulent. The increased virulence of the opaque variants is associated with increased expression of capsular polysaccharide and decreased expression of teichoic acids compared with the transparent phenotype.

Materials and Methods

Bacterial strains, growth conditions, and growth medium. Pneumococcal strains of types 6A, 6B, and 18C were originally obtained from blood isolates. Type 2 strain D39 and nonencapsulated mutants of this strain have previously been described [10, 17]. The capsular type was confirmed by the quellung reaction using type-specific antiserum (Dako, Carpinteria, CA). Bacteria were grown in a semisynthetic medium (C+Y medium, pH 8.0) at 37°C without shaking, as previously described [1]. Broth cultures were plated onto tryptic soy plates with 1% agar, onto which 5000 U of catalase (Worthington Biochemical, Freehold, NJ) was spread and incubated at 37°C in a candle jar extinction. Colony morphologies were determined under magnification and oblique transmitted illumination as previously described [1]. Unless otherwise stated, chemicals and reagents were purchased from Sigma (St. Louis).

Virulence studies in mice. Female BALB/c mice, 6–12 weeks of age, were obtained from Taconic Laboratories, Germantown, NY. Paired phenotypic variants were grown to mid-log phase ($A_{620} = 0.5$) and washed with PBS. They were adjusted to a density of 10^8 cfu/mL, and a volume of 0.2 mL was inoculated intraperitoneally into mice. Colonies were counted to confirm the quantity and phenotype of the inoculum. Mice were observed once daily for 20 days following inoculation, and time of death was recorded. Spleens of dead animals were recovered and cultured to determine the phenotype of the organism responsible for overwhelming infection. Differences in mean survival time between paired variants were analyzed by Fisher's exact test.

Quantitation of capsular polysaccharide. Phenotypic variants were grown to mid-log phase and harvested by centrifugation for 10 min at 1800 *g* to separate supernatant and whole cell fractions. The cells were resuspended in PBS at the original culture volume, sonicated for three 10-s intervals on ice, and stored at −20°C.

The quantity of capsular polysaccharide was determined by a sandwich ELISA previously described for bacterial capsular polysaccharide [18]. Type-specific rabbit antiserum (Dako) in a dilution of 1:5000 in 0.05 *M* Na_2CO_3 (pH 9.6) was fixed overnight at room temperature to 96-well plates (Greiner Labortechnik, Frickenhausen, Germany). Between each incubation step, the plate was washed five times with Tris buffer (10 m*M* Tris, 150 m*M* NaCl, 0.05% Brij, and 0.02% sodium azide). Samples of supernatant and sonicated cells were diluted across the plate and incubated at room temperature for 2 h with shaking. Standards consisted of purified capsular polysaccharide of the homologous type purchased from the American Type Culture Collection (Rockland, MD). After an additional five washes with Tris buffer, a mouse monoclonal antibody (MAb) to capsular polysaccharide of the same type (HASP 4 for type 6 and HASP 22 for type 18C) was added in Tris buffer at a concentration determined in pilot experiments, followed by incubation for 2 h at room temperature with shaking. After an additional five washes, an alkaline phosphatase–conjugated anti–mouse IgG (for HASP 4) or IgM (for HASP 22) was added at a dilution of 1:10,000 and incubated at room temperature for 2 h with shaking, and the A_{415} was determined as previously described [19]. Total cellular protein determination was carried out on sonicated cells using Micro BCA according to the manufacturer's directions (Pierce Chemical, Rockford, IL). The amount of capsular polysaccharide in the supernatant fraction was based on protein concentration in the cell sonicate fraction. Values obtained represent at least three determinations performed in duplicate.

Quantitation of total teichoic acid. The quantity of teichoic acid was determined by sandwich ELISA as per the method described for capsular polysaccharide quantitation with the following modification. A rabbit polyclonal antibody to C-polysaccharide at a dilution of 1:5000 was fixed onto microtiter plates. Standards consisted of purified lipoteichoic acid at a known concentration. Teichoic acid in supernatant or cell sonicate fraction was detected with either a mouse MAb to ChoP (HAS) or mouse MAb to C-polysaccharide (HASP 8), which does not bind to ChoP. The MAbs were used at a concentration determined in pilot experiments. Experiments were performed at least three times in duplicate.

Incorporation of [^{3}H]choline into teichoic acid and crude cell wall preparation. Teichoic acid was radiolabeled by adding [^{3}H]choline (New England Nuclear, Boston) to medium at a final concentration 0.5 mCi/mL. The C+Y growth medium also contained unlabeled choline at 5 mg/mL. Pneumococci grown to $A_{620} = 0.5$ were washed three times in an equal volume of PBS and resuspended in 1/20 the original culture volume. Pellets were resuspended in PBS, heated to 70°C for 20 min to inactivate autolysin, and sonicated as described above. An aliquot was removed for determination of incorporation of the label into whole cells and total cell-associated protein as described above. Crude cell walls were prepared by boiling the sonicated cells for 15 min in 5%

SDS as described [20]. After sedimentation of cell wall fragments for 10 min at 16,000 *g,* incorporation of the label was determined for both the sediment (cell wall–associated) and the soluble fraction (non–cell wall–associated). The crude cell wall preparation and SDS soluble fraction were also used in the ELISA to quantitate cell wall–associated and non–cell wall–associated teichoic acids.

DNA transformation. Chromosomal DNA was extracted from a PspA$^-$ mutant, WG 44.1, in which an erythromycin resistance marker had been inserted into the gene [16]. DNA was transformed into strain D39 according to a published procedure [21]. Transformants were selected in the presence of erythromycin at 1 mg/mL. Acquisition of the marker correlated with loss of expression of PspA from D39 on Western analysis using MAb Xi126.

Western blotting. Bacterial cell sonicates were adjusted to equal concentrations on the basis of cell density or protein content, and Western transfer and immunoblotting were performed as described [22]. Immunoblotting to detect PspA was carried out with MAb Xi126, which reacts with the protein in strain D39, at a dilution of 1:10 and detected with an alkaline phosphatase–conjugated anti–mouse IgG antiserum. Immunoblotting to detect pneumolysin was carried out with an antiserum to the toxin at a dilution of 1:1000 and detected with an alkaline phosphatase–conjugated anti–rabbit IgG serum. Immunoblotting to detect teichoic acid was carried out with a MAb to ChoP at a dilution of 1:10,000 and detected with an alkaline phosphatase–conjugated anti–mouse IgM serum.

Results

Relationship between colony morphology and intraperitoneal virulence. Transparent pneumococci have been shown to dominate the initial step in interaction with the host: nasopharyngeal colonization. Therefore, we examined the role of opaque pneumococci in subsequent events during pathogenesis. The colonization step was bypassed by administration of pneumococcus intraperitoneally in an animal model of sepsis using adult BALB/c mice. As shown in figure 1, when equal inocula of 10^7 cfu of opaque or transparent variants of type 2 isolate D39 were compared, there was a significantly greater mortality rate from organisms with the opaque phenotype (12/12 for opaque vs. 4/12 for transparent, $P < .001$). Similar results were obtained in unrelated isolates of type 6A (12/12 for opaque vs. 3/12 for transparent, $P < .001$) and 18C (6/8 for opaque vs. 1/8 for transparent, $P = .02$). In the case with the type 6A isolate, the intermediate-opacity phenotype was avirulent. An opaque revertant of this intermediate phenotype, however, was fully virulent (0/12 for intermediate vs. 12/12 for revertant, $P < .001$), supporting the relationship between colony morphology and virulence.

The cause of death was established by splenic culture. All mice dying following an inoculum of opaque organisms had organisms of the same phenotype as the inoculum recovered from their spleens. In contrast, all animals succumbing after receiving transparent type 2 or 6A organisms had pneumococci with a phenotype more opaque than the inoculum recovered from their spleens. These more opaque organisms had colony morphology similar but not identical to the intermediate phenotype. When the phenotype of recovered organisms differed from that of the inoculum, there was a delay averaging 2–3

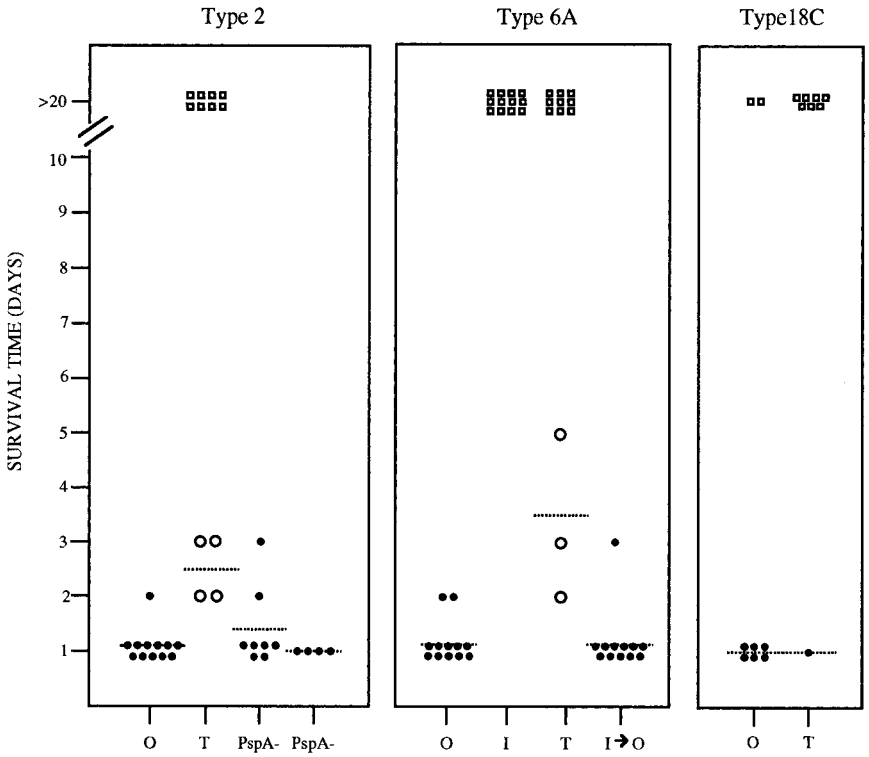

Figure 1. Relationship between virulence and colony morphology. Female adult BALB/c mice were given intraperitoneal inoculation with 10^7 cfu of isolate of type indicated. Spleens of dead mice were cultured to determine whether colony morphology resembled (●) or varied (○) from that of inoculum. Animals surviving >20-day observation period are shown as ◘. Variant phenotypes compared included opaque (O), intermediate (I), transparent (T), and opaque revertant of intermediate variant (I→O). PspA− refers to knockout insertion in gene for pneumococcal surface protein A constructed by transformation of DNA from defined mutant [15]. Average survival time of animals with positive splenic cultures is indicated by dashed line.

days in the time to death compared with the fully virulent opaque inoculum.

The role of known virulence determinants in the enhanced virulence of opaque pneumococci. Several bacterial factors implicated in pneumococcal virulence were compared in the opaque and transparent variants to account for the differences between phenotypes that were observed in mice.

The expression of pneumolysin and PspA were compared by Western analysis of whole cell lysates. There was no difference between variants in the amount or migration of pneumolysin, making it unlikely that this toxin is responsible for the greater virulence of the opaque phenotype. In the case of PspA, both opaque and transparent phase variants of D39 expressed PspA detected with MAb Xi126; however, the amount of protein expressed by equivalent numbers of cells was greater in the opaque variant (figure 2). To determine whether the increased expression of PspA in opaque pneumococci could account for findings in virulence studies, a mutation in its gene was transformed into the opaque variant of D39, and the loss of expression was confirmed by Western analysis (data not shown). The loss of PspA expression, however, did not affect colony morphology, and two separate $PspA^-$ transformants of the opaque D39 were not significantly different from $PspA^+$, opaque parent in intraperitoneal virulence (figure 1).

Quantitative differences in capsular polysaccharide were assessed using an ELISA in which the antigen is bound using type-specific antiserum and detected with a type-specific MAb. The amount of capsular polysaccharide present was calculated by comparison with standards consisting of same-type purified capsular polysaccharide of known weight and expressed relative to the amount of total cellular protein. Controls showed that the assay detected capsular polysaccharide of only the homologous type, and a nonencapsulated mutant showed no detectable material, as expected. The amount of capsular polysaccharide was measured in both the growth medium supernatant and the cell fraction.

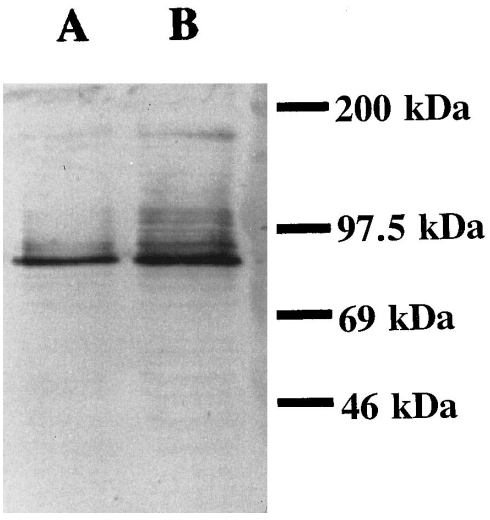

Figure 2. Relationship between pneumococcal surface protein A (PspA) expression and colony morphology. Whole cell lysates of equivalent numbers of cells of strain D39 with transparent (lane A) or opaque (lane B) phenotype were compared in Western analysis by separation on 10% SDS-PAGE and immunoblotting using monoclonal antibody Xi126 to PspA.

Results showed a mean of 2.4-fold more total capsular polysaccharide in the opaque than the transparent variant of the type 6A strain (figure 3). A type 6B isolate, not used in animal studies due to its lack of virulence, showed 5.6-fold more total capsular polysaccharide in the opaque than the transparent variant. Differences between phenotypes in the type 18C isolate were less marked (the opaque variant had 1.2-fold more total capsular polysaccharide than the transparent variant) and, unlike the other strains examined, not significantly different. Differences between intermediate and opaque variants of types 6A and 18C were not significant. Most of the difference between phenotypes was due to variation in amounts of capsular polysaccharide in the cell-associated fraction. The unavailability of a MAb to type 2 capsular polysaccharide prevented this assay from being used for strain D39. The quellung reaction of this strain, however, revealed an increased zone of reactivity in the opaque compared with the transparent variant. It was concluded that for strains virulent in mice, the increased virulence of the opaque pneumococci is associated with a higher amount of capsular polysaccharide.

Relationship between quantity of teichoic acid and colony opacity. Since phase variation in opacity occurs in the absence of encapsulation, differences in amount of capsular polysaccharide could not fully account for observations on colony morphology [1]. The question of whether differences in the quantity of the other polysaccharide structure on the cell surface, C-polysaccharide, could also vary was addressed using a similar ELISA. In this case, a MAb to ChoP was used, and the standards consisted of purified lipoteichoic acid of known concentration. A nonencapsulated mutant derived from strain D39 was used to demonstrate that the assay was unaffected by the presence of the capsule (figure 4A). Values were expressed relative to the amount of total cellular protein and fluctuated considerably from isolate to isolate but were consistent for analysis of variants of a given strain. In contrast to the results with capsular polysaccharide, the quantity of total teichoic acid was less in the opaque than the transparent variant for each of the strains examined. The ratio of total teichoic acid in the opaque compared with the transparent phenotype was 0.47, 0.26, and 0.48 for the nonencapsulated, type 6A, and type 18C strains, respectively. Similar findings were obtained for the type 6A isolate with a MAb to C-polysaccharide that does not react with the ChoP structure, showing that differences were not dependent on the ChoP structure (figure 4B).

Results with the ELISA were confirmed by comparing the incorporation of radiolabeled choline provided in the growth medium into teichoic acid, which is the only significant reser-

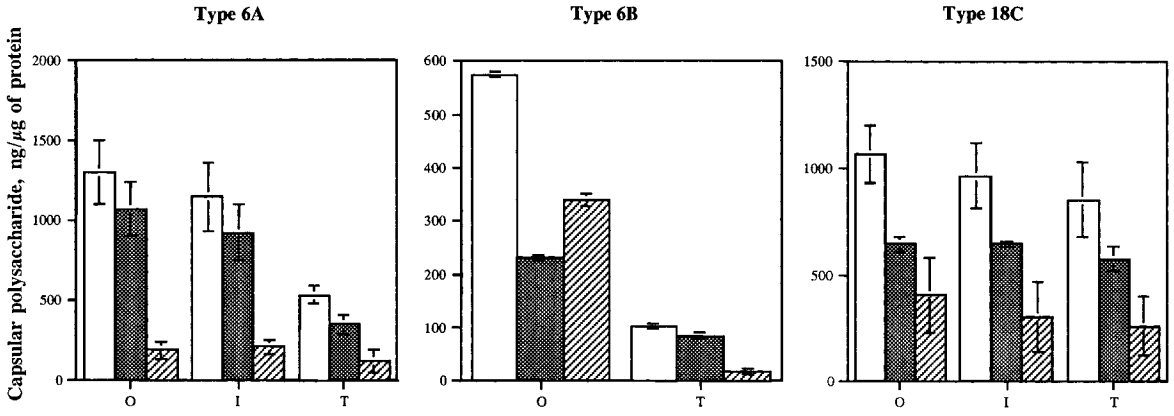

Figure 3. Relationship between quantity of capsular polysaccharide and colony morphology. Sandwich ELISA technique using type-specific monoclonal antibodies was used to determine amount of capsular polysaccharide in growth medium supernatant (hatched bars) or cell-associated fractions (dark gray bars) and combined total (open bars). Values were calculated by comparison with standards consisting of purified capsular polysaccharide of same type and are expressed as ng/μg of total cellular protein ± SE. Opaque (O), intermediate (I), or transparent (T) variants of isolate of type indicated were compared.

voir for choline. The amount of [^{3}H]choline incorporated into opaque cells was 0.24- and 0.65-fold that of the transparent variant of the nonencapsulated and type 6A strains, respectively (figure 5). Most of this difference was attributable to the cell-associated fraction.

To determine whether a difference in amount of total teichoic acid was caused by differences in the lipoteichoic acid or cell wall–associated teichoic acid, cells grown in [^{3}H]choline were fractionated after treatment of sonicated cells at 100°C in 5% SDS. As expected, crude cell walls in the SDS-insoluble frac-

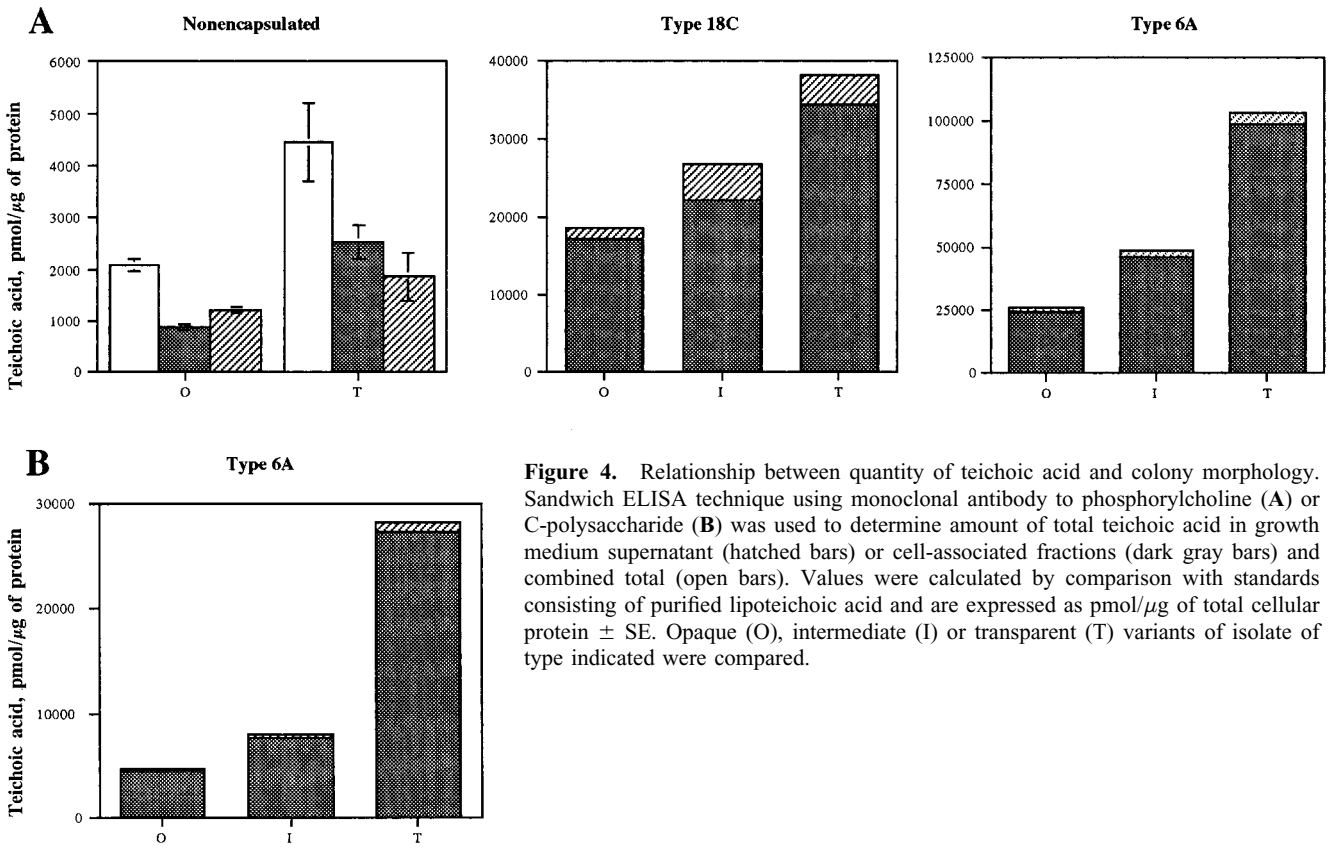

Figure 4. Relationship between quantity of teichoic acid and colony morphology. Sandwich ELISA technique using monoclonal antibody to phosphorylcholine (**A**) or C-polysaccharide (**B**) was used to determine amount of total teichoic acid in growth medium supernatant (hatched bars) or cell-associated fractions (dark gray bars) and combined total (open bars). Values were calculated by comparison with standards consisting of purified lipoteichoic acid and are expressed as pmol/μg of total cellular protein ± SE. Opaque (O), intermediate (I) or transparent (T) variants of isolate of type indicated were compared.

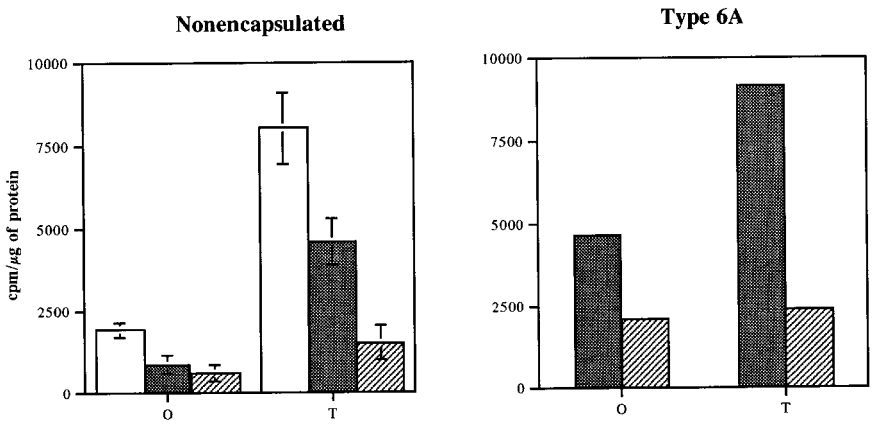

Figure 5. Incorporation of choline in pneumococcal variants. Opaque (O) or transparent (T) variants of strain indicated were grown in presence of [^{3}H]choline. Incorporation of radiolabel was determined in whole cells (open bars) and in sonicated cells treated at 100°C in 5% SDS and separated into non–cell wall–associated (hatched bars) and cell wall–associated fractions (dark gray bars). Values are cpm/μg of total cellular protein ± SE.

tion contained >80% of the radiolabel (figure 5). Differences between phase variants for the nonencapsulated and type 6A strains could be attributed to differences in incorporation of choline into the SDS-insoluble fraction containing the cell wall–associated teichoic acid. ELISA detected teichoic acid in both SDS-soluble (lipoteichoic acid) and insoluble fractions and showed similar differences between phenotypes (data not shown).

Further characterization of the teichoic acids was carried out by Western analysis (figure 6). Whole-cell lysates of opaque, intermediate, and transparent organisms loaded in equal amounts on the basis of protein content were separated on 15% SDS-PAGE and immunoblotted with a MAb to ChoP. This technique detects lipoteichoic acid, which appears as a ladder-like array of bands similar to controls with purified lipoteichoic acid (data not shown). These bands represent chains with varying numbers of repeat units [23]. There was no difference between variants in the rate of migration of each chain or in the average chain length.

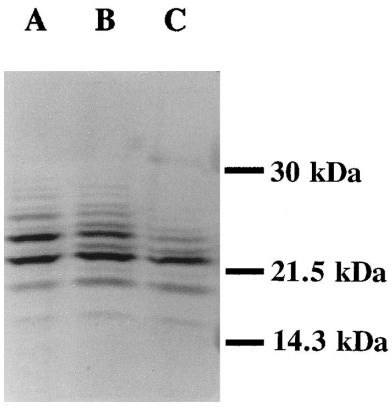

Figure 6. Western analysis of teichoic acid. Whole cell lysates of transparent (lane A), intermediate (lane B), and opaque (lane C) variants of type 6A isolate were adjusted for equal loading on basis of total cellular protein concentration, separated on 15% SDS-PAGE, and immunoblotted with monoclonal antibody to phosphorylcholine.

The role of teichoic acid in the enhanced virulence of opaque pneumococci. The comparison of variants left it unclear whether the increased virulence of the opaque phenotype is caused by a higher amount of capsular polysaccharide, lower amount of teichoic acid, or both. Insight into this question was gained by analysis of the more opaque phenotype selected during systemic infection. Splenic variants differing in colony morphology from the pneumococci given in the inoculum were characterized. Those recovered after inoculation of the transparent type 2 pneumococcus had a zone of capsule detected by quellung reaction larger than that of the inoculum and similar to that of the opaque variant. The more opaque variant recovered after an inoculum of transparent type 6A organisms, however, was not different in amount of capsular polysaccharide from the inoculum, as measured by ELISA (figure 7A). In contrast, for both the type 2 and 6A isolates, following inoculation with transparent organisms, three variants recovered from spleens were analyzed and each had significantly less (type 2, 0.62- and 0.52-fold; type 6A, 0.62-fold) teichoic acid than the transparent inoculum (figure 7B). This suggested that the opacity phenotype and the amount of cell-associated teichoic acid may be critical to the ability of the pneumococcus to cause systemic infection.

Discussion

S. pneumoniae undergoes intrastrain phase variation in two aspects: colony morphology and virulence. The pneumococcus varies spontaneously between a relatively avirulent phenotype adapted for intranasal carriage and a more virulent phenotype, which colonizes inefficiently. The phenotypes are easily distinguished by differences in colony morphology (i.e., opacity). In this study, there was a relationship between colony morphology and virulence in an animal model of sepsis.

Comparison of the opaque and transparent variants revealed quantitative differences in the amount of PspA, capsular polysaccharide, and cell wall–associated teichoic acid correlating with colony opacity.

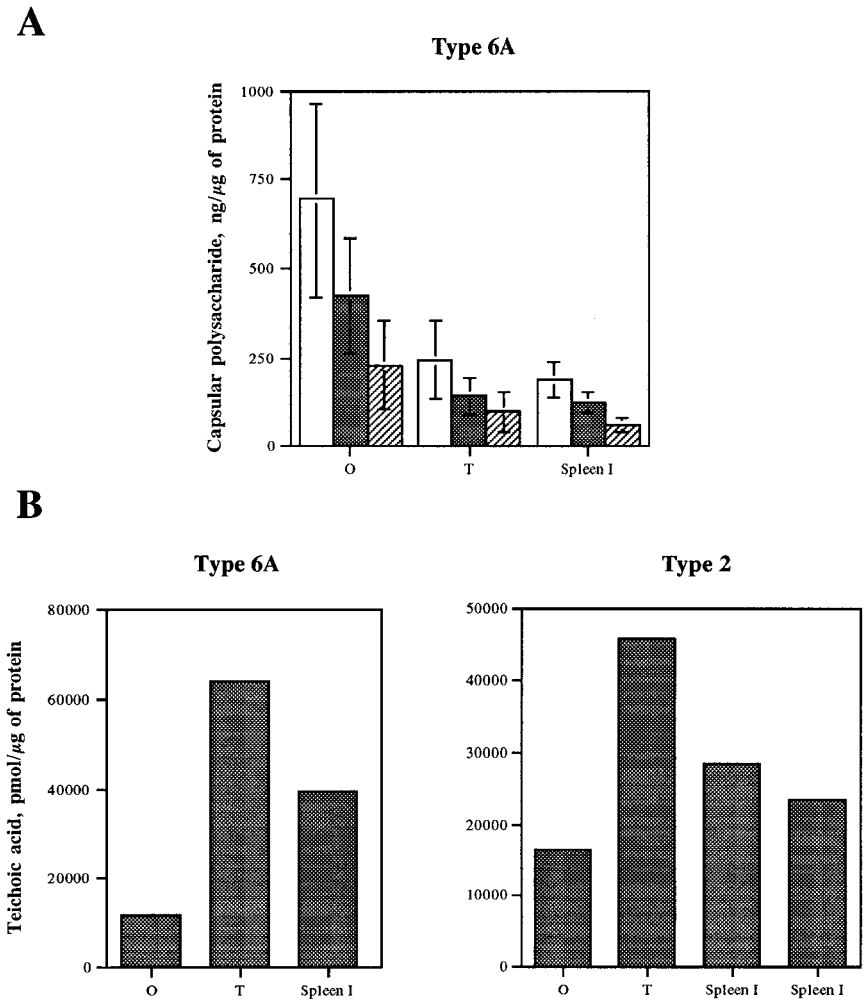

Figure 7. Characteristics of pneumococcal isolates recovered from animals. BALB/c mice were inoculated intraperitoneally with transparent variants of strain types 2 and 6A as indicated. Pneumococci recovered from splenic cultures of dead animals were different from inoculum in colony morphology and had more opaque phenotype (spleen I). Variants recovered from splenic cultures were compared with opaque (O), intermediate (I), and transparent (T) phase variants of same strain for quantity of capsular polysaccharide (**A**) and cell-associated teichoic acid (**B**) using ELISA. Values for capsular polysaccharide were determined for growth medium supernatant (dark gray bars) or cell-associated fractions (stippled bars) and combined total (open bars) and are expressed as ng/μg of total cellular protein $\pm$ SE. Values for total teichoic acid are expressed as geometric mean of 2 determinations in pmol/μg of total cellular protein.

Several cell-surface structures were analyzed to define which factor determines differences in colony opacity. Opaque variants contained higher amounts of PspA. However, mutants lacking PspA also undergo variation in phenotype, making it unlikely that this protein determines colony morphology. Likewise, the phenomenon of phase variation in colony morphology occurs in the absence of capsular polysaccharide (i.e., in nonencapsulated strains). Thus, capsular polysaccharide also is not necessary for variation in colony opacity. Phenotypic variation was, however, consistently associated with at least 2-fold differences in total teichoic acid content. Therefore, it is possible that this structure is responsible for phase variation in colony morphology. This could not be confirmed by mutational analysis because mutants in the pneumococcal teichoic acid have not been described. Differences in teichoic acid content may also affect the cell-surface expression of the multiple choline-binding proteins anchored to ChoP. With the exception of PspA, choline-binding proteins have been found in greater amounts on transparent pneumococci, correlating with the increased total teichoic acid content associated with this phenotype [11].

Previously, MacLeod and Krauss [14] demonstrated interstrain variation in capsular polysaccharide content. Amounts of capsular polysaccharide of individual strains also vary, depending on the growth conditions [24]. The current study showed intrastrain variation in the quantity of capsular polysaccharide correlating with phase variation in colony morphology. An earlier report suggested that variation in colony opacity was not associated with differences in amount of capsular polysaccharide [1]. This finding, however, was based on the observation of variation in colony opacity in nonencapsulated strains and a less-sensitive ELISA technique. The availability of serotype-specific MAbs allowed for more precise quantitation using ELISA in the present study. Preliminary findings by immunoelectron microscopy of the type 6A variants demonstrated a thicker capsule in the opaque than the transparent phenotype, as predicted in this report (Sorensen US, personal communication).

In contrast to trends in the amounts of capsular polysaccharide, the transparent phenotype contained 2.1- to 3.8-fold more total teichoic acid. Prior work by Sorensen et al. [24] produced evidence of interstrain differences in amount of C-polysaccharide as shown by thickness of the layer on electron micrographs. The current study documented intrastrain variation in teichoic acid content. These differences were accounted for primarily by variation in the cell wall–associated form of teichoic acid and were demonstrated using two methods: immunologic (ELISA) and nonimmunologic ([^{3}H]choline incorporation). Western analysis showed that the lipoteichoic acid appears to be structurally similar in opaque and transparent variants. Structural analysis of differences in cell wall–associated teichoic acid was beyond the scope of this study; however, in the pneumococcus, the repetitive units of the lipoteichoic acid and cell wall–associated teichoic acid are identical. The cell wall–associated teichoic acid is covalently attached to muramic acid residues in the peptidoglycan, and only a fraction of these residues appears to be linked to a teichoic acid chain [25]. The major differences in the amount of teichoic acid between phenotypes could have been a consequence of different proportions of muramic acid residues with an attached teichoic acid chain. It has been suggested previously that this proportion is variable [25].

With respect to the overall structure of the pneumococcus, it has been proposed that the capsular polysaccharide and cell wall are covalently linked, although the precise nature of this linkage has not been defined [26]. Results showing an inverse relationship between quantities of capsular polysaccharide and teichoic acid supported the evidence that there may be an interdependence in the surface expression of the two major carbohydrate structures of the pneumococcus. The inverse relationship between amounts of capsular polysaccharide and teichoic acid with colony morphology is summarized for 2 unrelated isolates in table 1. Variation in the ratio of capsular polysaccharide to teichoic acid appears to be consistent from strain to strain. The details of the molecular mechanism that mediates phase variation and regulates expression of these two complex structures (capsular polysaccharide and teichoic acid) remain unresolved. A genetic element that affects the frequency of phase variation in colony opacity has been described [27].

Various surface structures have been proposed as virulence factors in the pathogenesis of pneumococcal disease. Our studies examined a number of these in relation to phase variation and the enhanced virulence of opaque phenotypes. Mutagenesis of PspA, which is expressed in higher amount in the virulent, opaque pneumococci, was not associated with a difference in virulence compared with the parent strain. Differences in the expression of PspA, therefore, did not appear to account for the enhanced virulence of opaque pneumococci.

The amount of capsular polysaccharide has long been known to be an important factor in virulence. MacLeod and Krauss [14] demonstrated a correlation between intraperitoneal virulence in adult mice and quantity of capsular polysaccharide among different strains of the same serotype. That study showed 1.5- to 4.3-fold more capsular polysaccharide in the more virulent isolate of each type. In the present study, an intrastrain variation of 1.2- to 5.6-fold higher quantities of capsular polysaccharide in the virulent, opaque phenotype was shown. In each of these studies, capsular polysaccharide was quantitated for organisms grown in vitro and may not accurately reflect amounts expressed in vivo.

Table 1. Summary of comparisons between phenotypic variants in quantity of surface polysaccharides.

	Opaque	Intermediate	Transparent
Type 6A			
Capsular polysaccharide (pmol/μg of protein)*	1360	1201	563
Teichoic acid (pmol/μg of protein)	26,900	49,794	105,147
Capsule/teichoic acid	0.0505	0.0241	0.0054
Type 18C			
Capsular polysaccharide (pmol/μg of protein)*	677	613	542
Teichoic acid (pmol/μg of protein)	18,651	27,422	38,543
Capsule/teichoic acid	0.0363	0.0224	0.0141

* Capsular polysaccharide quantity was converted from ng/μg of protein to pmol/μg of protein on basis of molecular weights of repeating polymer units of capsule: type 6A, 958 g/mol; type 18C, 1577 g/mol. All values reflect total quantities (supernatant + cell fraction).

Our findings demonstrated that the pneumococcus phase varies between an avirulent form, better adapted for carriage, and a virulent form, deficient in its ability to colonize the nasopharynx. Similar variation between phenotypes adapted for colonization or systemic infection have been reported among other encapsulated species that reside in the human nasopharynx and frequently cause invasive infection. In both *Neisseria meningitidis* and *H. influenzae* type b, there is phase variation between more- and less-virulent phenotypes that is based on changes in lipopolysaccharide structure and quantity of capsular polysaccharide [18, 28-31]. It remains unclear, however, why an organism residing on the mucosal surface of the nasopharynx, usually without causing disease, would vary to a more-virulent phenotype, potentially fatal to its host. Perhaps the shifting between phenotypes contributes to the ability of the organism to persist for protracted periods on the mucosal surface. The higher amounts of capsular polysaccharide and decreased teichoic acid residues noted in the opaque pneumococcus may protect the organism from deposition of complement and phagocytosis, although constitutive expression of such characteristics may not be optimal for long-term nasopharyngeal carriage. The increased teichoic acid of the transparent organism might be necessary for colonization, because this structure anchors multiple choline-binding proteins that may function in

attachment and binding to epithelial cell receptors directly via the ChoP structure [3, 11].

In the pathogenesis of invasive pneumococcal disease, the precise role of teichoic acid, unlike capsular polysaccharide, is not completely understood. Our study suggested that teichoic acid is particularly important in the ability of the organism to colonize the nasopharynx; organisms with an increased amount of teichoic acid are favored in adherence to epithelial cells and colonization. Results from the current study demonstrated that teichoic acid is also a factor in the ability of the pneumococcus to cause sepsis. Following intraperitoneal challenge of adult mice, only those organisms with lower amounts of teichoic acid were successful in causing overwhelming infection. This may be because teichoic acid binds to C3b, activating the alternative pathway [32, 33]. In addition, natural antibody to the ChoP epitope exists in the human bloodstream [34]. There may be a threshold for the density of teichoic acid residues allowing for evasion of host clearance mechanisms. An amount of teichoic acid that is required for colonizing the mucosal surface might not allow survival within the bloodstream, where the deposition of antibody and complement may be more efficient. This report, therefore, provides additional evidence that teichoic acid provides more than just structural integrity to the pneumococcal cell wall; it is also an important factor in the interaction of the pneumococcus with its human host. Finally, these observations may be of general significance to respiratory tract infection in humans, as the ChoP structure found on the pneumococcal teichoic acid is now known to be common to other major bacterial pathogens infecting this site [7].

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