INFECTION AND IMMUNITY, July 1981 p. 59-66
0019-9567/81/070059-08$02.00/0

Vol. 33, No. 1

Increased Virulence of *Neisseria meningitidis* After In Vitro Iron-Limited Growth at Low pH

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Received 2 March 1981/Accepted 21 April 1981

At low pH (6.6) and under conditions of iron limitation, *Neisseria meningitidis* group B (strain SD1C) exhibited an atypical outer membrane protein profile and an increased relative virulence for the mouse. Cells grown in a buffered medium were effectively deprived of iron by the addition of ethylenediamine-diorthohydroxyphenylacetate. The pH of the medium selected for characteristic colonial morphologies: type M3 predominated at pH 6.6, and type M5 predominated at pH 7.7. A mixed population of M1, M3, and M5 colonies was observed at pH 7.2. Isolated outer membrane proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and surface exposed proteins were labeled by the [^{125}I]lactoperoxidase method and subsequently identified by autoradiography. Cells grown at pH 6.6 elaborated a major outer membrane protein (protein III; molecular weight, 69,000), which was also present in the outer membrane of iron-limited cells grown at pH 7.2. At pH 7.2 in an iron-sufficient medium, protein III was present only in small quantities in sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. A study of the relative virulence (50% lethal dose) of the meningococcus for C57/BL mice revealed that iron-limited cells grown at low pH had an increased relative virulence 1,200-fold (50% lethal dose, 4.0 CFU) greater than that of cells grown in the same medium but at pH 7.2 and with sufficient iron. These studies indicate that pH and iron can be important factors in the determination of meningococcal virulence.

The bacterial surface interacts directly with host defense mechanisms during the infection process. The composition of the surface alone can determine to a degree the relative virulence of a pathogen. In the healthy host, meningococci are normally restricted to the surface of epithelial cells that line the nasopharynx; however, during systemic infection meningococci are capable of growth in a variety of other environmental niches within the body, e.g., cerebrospinal fluid, blood, and serous fluid. It is a well-known feature of microbes to adjust their metabolism or biochemical composition in response to changing nutritional or physical environmental conditions (10, 26, 30, 33).

In response to changes in carbon sources, growth temperature, and availability of iron, *Escherichia coli* surface protein profiles on polyacrylamide gels are changed substantially (25, 29). Moreover, among the neisseriae, outer membrane profiles of gonococci are altered in response to changing pH of growth or to iron limitation (27, 28), and the compositioin of lipopolysaccharide changed when cultural conditions of *Neisseria sicca* were altered (26).

Several investigations have focused on capsular and noncapsular antigenic determinants on the meningococcal surface (12, 27, 31, 34, 39, 44). It is evident that certain noncapsular antigenic surface proteins associated with specific meningococcal serotypes are more frequently found in disease-associated strains (12). Therefore, it seems reasonable to postulate that major changes in the composition of the outer membrane proteins of meningococci could influence the relative virulence of this bacterium. In this report we present evidence that manipulation of pH and iron availability in concert not only can change the outer membrane protein profile but also can greatly increase the relative virulence of this organism.

MATERIALS AND METHODS

Organism. *Neisseria meningitidis* group B (strain SD1C) was obtained from the Neisseria Repository, NAMRU, University of California, Berkeley. Maintenance of stock cultures and routine examinations for purity were described elsewhere (8).

Cultural conditions. Frozen working cultures (−70°C) were thawed at 37°C and streaked onto Mueller-Hinton (M-H) agar (Difco Laboratories, Detroit, Mich.) plates which were then incubated for 16 to 20 h in a candle extinction jar (37°C, 100% relative hu-

midity). In some experiments, M-H medium was buffered with 25 mM mono-tris(hydroxymethyl)-aminomethane-maleate (Sigma Chemical Co., St. Louis, Mo.) and titrated with 4 M NaOH to the desired pH value. Iron limitation during growth was achieved by the addition of the iron chelator ethylenediamine-diorthohydroxyphenylacetate (EDDA; ICN Pharmaceuticals Inc., Plainview, N.Y.) to the medium at a final concentratioin of 7 μg/ml (1).

For the experiments on the effect of pH on growth, colonies from the M-H agar plates described above, served as the inoculum for a sterile M-H broth (10 ml) which was subsequently incubated at 37°C with shaking (100 rpm) for 9 h. A fixed volume (0.5% [vol/vol] final concentration) of this culture was then transferred to buffered M-H broths at preset pH values. In all experiments, starting pH values were determined after inoculation of the test media. Growth was monitored spectrophotometrically (Spectronic 20, Bausch & Lomb, Inc., Rochester, N.Y.) and by determination of viable cells.

Colony morphology. Meningococci from appropriate broth cultures were plated onto buffered M-H agar. After 28 h of incubation in a 37°C candle extinction jar, colonies were analyzed by means of a dissection light microscope. Photographs of colonies were taken by using a high-intensity side-arm illuminator as described previously (4).

Isolation of outer membrane proteins. Cells were harvested from M-H agar plates (see above) and suspended in 7.5 ml of test medium. This suspension was the inoculum for a final 1,500-ml broth of the same medium. Cultures were incubated until late-logarithmic phase (optical density at 600 nm, 0.6), except for cultures in media with EDDA which were incubated until division in the culture ceased due to iron limitation (optical density at 600 nm, 0.2 to 0.4).

Cells were harvested by centrifugation (4°C) at 8,000 × *g* for 10 min. Suspensions of cells were twice extruded from a precooled French pressure cell (F. S. Carver Inc., Summit, N.J.) maintained at 12,000 to 16,000 lb/in^2. Cell debris was discarded (12,000 × *g*, 10 min), and the supernatant solution was subjected to three sequential centrifugations each at 75,000 × *g* (mean force) for 1 h (Beckman L5-65 ultracentrifuge; 60 Ti rotor). The cytoplasmic membrane was selectively solubilized with 1% (wt/vol) sodium-*N*-lauroylsarcosinate (Sigma) as previously described (11, 37).

Iodination. The ^{125}I labeling of intact meningococci was similar to that previously described for gonococci (16). Broth cultures of meningococci were centrifuged at 8,000 × *g* for 10 min, and the pelleted bacteria were suspended in 0.15 M sodium phosphate-buffered saline, pH 7.4. The concentration of the suspension was adjusted so that a 10-fold dilution had an optical density at 550 nm of 0.6 (Gilford 250 spectrophotometer; Gilford Instrument Labs, Oberlin, Ohio). The bacterial suspension (0.5 ml) was warmed to 30°C; then 50 μl of lactoperoxidase solution (1.0 mg/ml; Sigma) and 50 μl of carrier-free Na^{125}I (1 mCi/ml; New England Nuclear Corp., Boston, Mass.) were added. The reaction was started by the addition of 0.01 M H_2O_2 (50 μl) and maintained by further additions of peroxide at 2.5, 5.0, and 7.5 min. After 10 min, cysteine (5 mM final concentration) in phosphate-buffered saline was added, and the mixture (10 ml) was centrifuged at 15,000 × *g* for 20 min. The labeled meningococci were washed three more times in phosphate-buffered saline (1.0 ml) before samples were removed for protein analysis (23) and for gamma counting (model 5375; Packard Instrument Co., Inc., Ill.).

Electrophoresis. Polyacrylamide slab gel electrophoresis was carried out essentially by the method of Lugtenberg et al. (24). Slabs (0.75 mm thick) had an 11-cm running gel (11% [wt/vol] acrylamide; Eastman Kodak Co., Rochester, N.Y.) and a 3-cm stacking gel (3% [wt/vol] acrylamide). Buffers and sample (1.0 mg of protein per ml) were prepared as previously described (24), except that 0.05% bromophenol blue tracker dye was used. The gels were prerun for 2 h at 20°C at a constant current of 20 mA per gel. After sample application (20 μl per slot) a 20 mA constant current was applied, and electrophoresis proceeded until the tracking dye migrated to the bottom of the gel. Gels were stained for 1 h in a solution of 0.4% (wt/vol) Coomassie brilliant blue R-250 (ICN Pharmaceuticals Inc., Plainview, N.Y.), 15% (vol/vol) glacial acetic acid, and 50% (vol/vol) methanol. After destaining in a solution of 10% methanol and 5% glacial acetic acid, gels were placed on Whatman no. 3 filter paper and placed in a Bio-Rad gel-drying apparatus.

For autoradiography, dry gels were placed in contact with Kodak X-Omat MA X-ray film for 48 to 72 h (−70°C) before development.

Virulence studies. The procedure followed was that of Holbein et al. (18). Mice (male C57/BL, 20 to 25 g) were fed and watered ad libitum throughout all experiments. Groups of mice (six per group) were pretreated by injecting intraperitoneally 0.5 ml of iron dextran (250 mg of Fe per kg body weight; Dextran Products Ltd., Scarborough, Ontario, Canada) immediately before intraperitoneal injection with 0.5 ml of a bacterial suspension. Infecting doses ranged from 1 to 10^6 colony-forming units per mouse. Controls received either the highest number of bacteria with no iron dextran or iron dextran alone. *N. meningitidis* SD1C is incapable of in vitro growth on media in which iron dextran is the sole source of iron (1). Experiments lasted 72 h, at which time the 50% lethal doses were derived (32) in terms of colony-forming units.

RESULTS

In the initial experiments, the range of pH values was determined over which cells could initiate growth in unmodified Mueller-Hinton broth. A simple pH adjustment produced media ranging in pH from 5.8 to 8.4. After the inoculation of such media with a standard number of cells (10^7 colony-forming units, final), both the increase in viable cells and the changing pH were monitored over time. Cells were able to initiate and maintain growth in cultures with starting pH values within the range 6.2 to 8.2 (Fig. 1). Both the initial rates of cell division and the initial pH in most cultures changed during

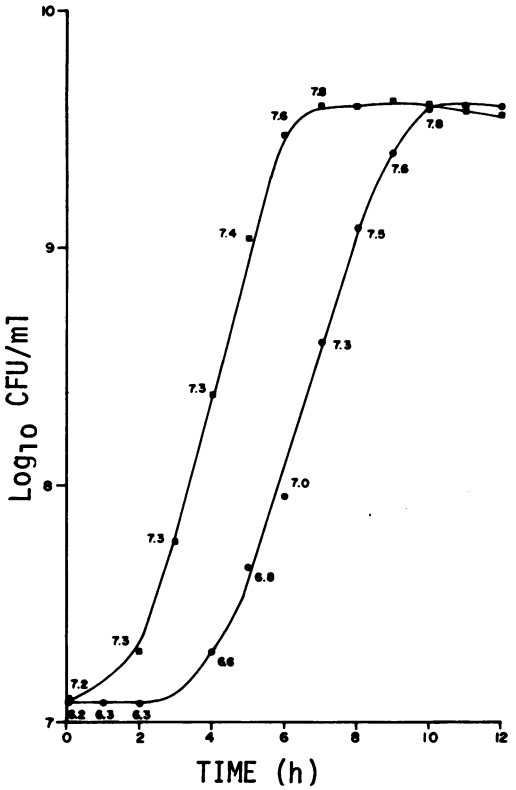

FIG. 1. *Growth of N. meningitidis in M-H broth in which the pH at the time of inoculation was 6.2 (●) or 7.2 (■). Cells grown in M-H broth at initial pH of 8.2 (not shown), exhibited a prolonged lag phase accompanied by a concomitant decrease in pH before exponential growth.*

growth. Such changes were especially dramatic at the extremes of the pH range. Cells were capable of manipulating the pH of the medium either upward, in the case of those in media at low initial pH, or downward, in cultures in which the initial pH was high. In cultures initiated at pH 6.4, for example, the cells were able to change their environment as much as 0.1 pH units per cell doubling. The rate of cell division in the cultures at initial pH values of 6.2 and 8.2 remained extremely slow until the changing pH value had reached 6.6 and 8.0, respectively, at which time cell division proceeded exponentially at similar rates irrespective of the initial pH.

To determine the effect of a fixed pH on cell growth, a buffered M-H medium, in which cells could not affect the pH during growth, was used. To achieve this, M-H broth was buffered with tris(hydroxymethyl)aminomethane-maleate which did not, itself, stimulate or inhibit the growth of cells in M-H broth; therefore, the effects on growth were due to pH alone. The results in Fig. 2 show that in an environment in which cells could not change the pH, the absolute low and high pH values at which cells would grow were 6.6 and 7.7, respectively. At these extremes, the rates and extents of growth were similar, although somewhat reduced in comparison with those at pH 7.2, the center of the range. Outside these extremes cell division started, but growth was very limited and could not be maintained. Surprisingly, cells at pH 5.8 survived for prolonged periods (>4 h), but were incapable of cell division at that pH.

The importance of environmental conditions in determining colonial morphology is well recognized (3). To determine the effect of pH on colonial morphology in our experiments, M-H agar was buffered at the appropriate pH as described above. The effect of pH on colonial morphology was particularly pronounced at the

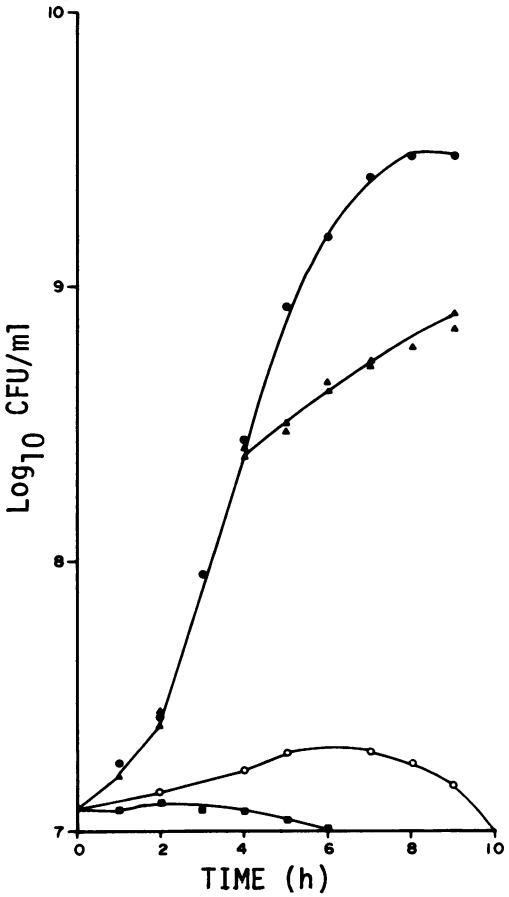

FIG. 2. *Growth of N. meningitidis in buffered M-H broth at pH 5.8 (■), 6.2 (○), 6.6 (▲), 7.2 (●), and 7.7 (△).*

pH extremes, 6.6 and 7.7. Three colonial types were observed routinely (Fig. 3) which closely resembled types M1, M3, and M5 previously described by this laboratory (9). Quantitation of the colonial types on media at pH 6.6, 7.2, and 7.7 revealed that the pH of the medium at the extremes selected for characteristic colonial morphologies (Fig. 4), whereas the colonial types at the center of the range (pH 7.2) were mixed.

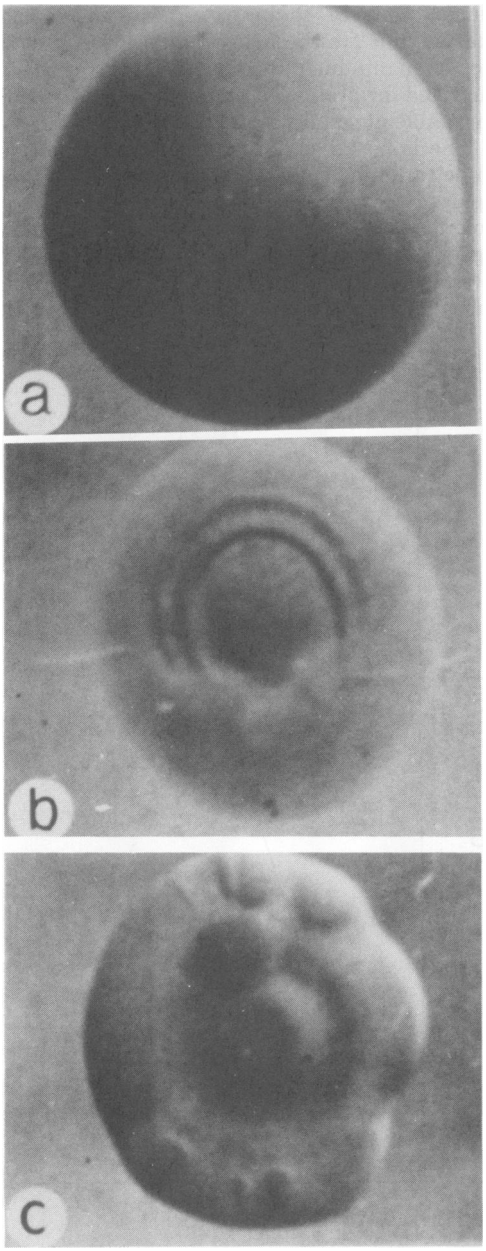

FIG. 3. *Three colonial morphologies of N. meningitidis typical after 28 h growth in buffered M-H medium at pH 7.2 (a), 6.6 (b), and 7.7 (c) (× 50).*

Analysis of isolated outer membrane protein profiles after sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that, indeed, there had been changes that were directly attributable to the pH of the buffered growth medium (Fig. 5). The principal outer membrane protein, protein I (36,000 molecular weight), remained unchanged irrespective of the pH of the growth medium. However, growth at pH 6.6 (as compared to growth at pH 7.2) resulted in the elaboration of an outer membrane protein of 69,000 molecular weight (protein III) and a concomitant decrease in the higher-molecular-weight (76,000) protein II.

A recent report from this laboratory (37) has shown that the same strain of meningococcus grown in a defined medium deficient in iron at pH 7.4 also elaborated relatively large quantities of protein III when compared with cells grown in iron-sufficient medium. Iron starvation in M-H broth was achieved by the additioin of the iron chelator EDDA to the medium as we have reported previously (2). The result of iron starvation in the complex medium at pH 7.2 was the appearance once again of the same outer membrane protein III (Fig. 5). The predominance of protein III when cells were grown at pH 6.6 or iron starved prompted us to attempt the combination of both iron-limited growth and low pH (6.6). Grown under these conditions, not only

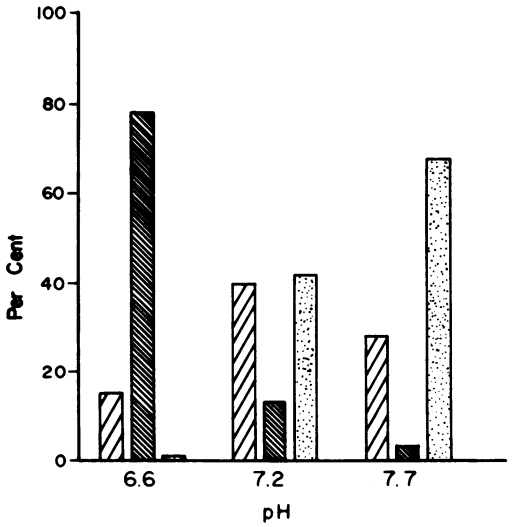

FIG. 4. *Relative distribution of N. meningitidis colonial types a (▨), b (▩), and c (▩), after 28 h of incubation in buffered M-H media set at pH 6.6, 7.2, or 7.7. Two hundred isolated colonies grown at each of the three pH values were counted.*

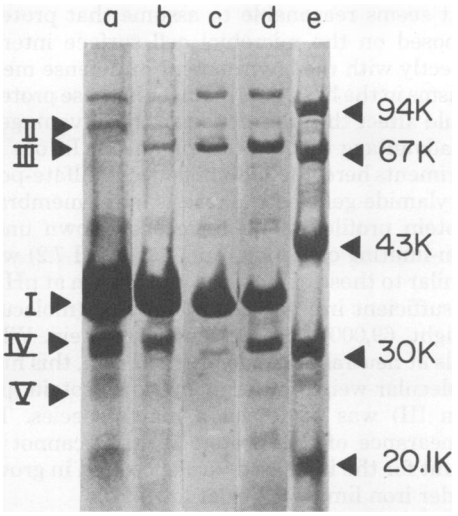

FIG. 5. *Polyacrylamide gel electrophoretic patterns of the outer membrane proteins of N. meningitidis grown at different pH values and in the presence and absence of the iron chelator EDDA. Protein bands were stained with Coomassie brilliant blue. Profiles are from cells grown at pH 7.2 (a), pH 6.6 (b), pH 7.2 with EDDA (c), and pH 6.6 with EDDA (d). Proteins of known molecular weight were also separated (e).*

did cells elaborate high quantities of protein III, but also another protein (IV) appeared that had a molecular weight of 31,000.

In view of the striking changes in the outer membrane protein profiles of the meningococci induced by low pH and iron starvation, we were interested to see whether such changes might correlate with a changed virulence for mice. A direct role for proteins III and IV in host-parasite interactions would require that they be oriented and exposed to the outside of the cell. To determine the orientation of the individual proteins in the outer membrane, the [^{125}I]lactoperoxidase labeling technique (16) was used. With this method only the aromatic amino acid sidechains exposed on the surface of the organism are iodinated. The autoradiographs from labeled outer membrane profiles (Fig. 6) clearly indicate that proteins I, III, and IV were oriented toward the outside of the cell. Therefore, such proteins could be expected to interact directly with host cells and body fluids.

The growth of cells at pH 7.2 or 7.7 resulted in the appearance of another major iodinated protein (protein V; molecular weight, 28,000). Protein V never appeared in radioautographs of cells grown at pH 6.6 whether or not cells had been starved for iron (Fig. 6).

Cells grown under the various conditions described above were then tested for their relative virulence in mice by a method described by Holbein et al. (17, 18). The results of our virulence testing are shown in Table 1. The virulence index increased 1,200-fold when cells were grown in vitro at low pH with iron limitation. This enhanced virulence correlated directly with the appearance in the outer membrane of proteins III and IV and with the shift to colonial type M3.

DISCUSSION

We have presented evidence here that the combined effects of iron starvation and low pH during growth of *N. meningitidis* greatly increased (1,150-fold) the relative virulence of this microorganism for mice. The effects on virulence

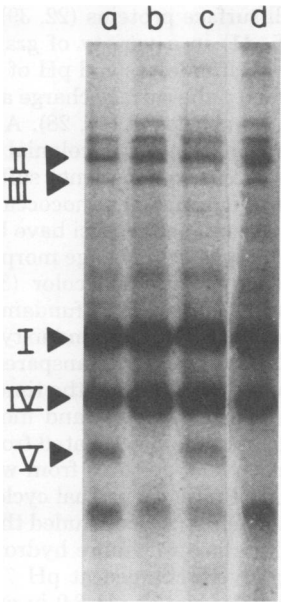

FIG. 6. *Radioautograph of ^{125}I-labeled outer membrane proteins of meningococci grown at pH 7.2 (a), pH 6.6 (b), pH 7.7 (c), and pH 6.6 with EDDA (d).*

TABLE 1. *Virulence for mice of N. meningitidis cultured in vitro at fixed pH and under conditions of iron limitation*

Growth conditions	LD_{50}[a]	P[b]	VI[c]
pH 7.2	$4.6 (\pm 0.7) \times 10^3$		1
pH 7.2 plus EDDA	$5.0 (\pm 0.9) \times 10^2$	0.95	9
pH 7.7	$3.7 (\pm 0.8) \times 10^2$	0.95	12
pH 6.6	$6.8 (\pm 2.7) \times 10^1$	>0.95	68
pH 6.6 plus EDDA	$4.0 (\pm 4.1) \times 10^0$	>0.99	1150

[a] LD_{50}, Mean 50% lethal dose of meningococci on triplicate sets of six C57/BL mice (20 to 25 g) per dose by the method of Reed and Muench (32).

[b] P, Probability level of significance (F-ratio one-tailed test).

[c] VI, Virulence index, defined as LD_{50} (pH 7.2)/LD_{50} (test).

of either depleted iron or low pH, separately, were relatively minimal. The growth at low pH in sufficient iron increased the relative virulence index only 68-fold, whereas iron starvation at neutral pH increased the relative virulence only 9-fold. However, such small virulence increases suggest that the much greater increase in virulence when low pH and low iron are combined is brought about by a combination of more than one phenotypic change in the microorganism.

This laboratory has previously reported seven different colony types (4, 9) for meningococci. In this report, we clearly demonstrated that low pH did, indeed, select for characteristic M3 colonial morphology. Differences in colonial morphology have been associated with differences in lipopolysaccharide (36), antigenic composition (12, 13), cell surface proteins (22, 39), and virulence (5, 35, 41) in a variety of gram-negative bacteria. Both iron levels and pH of the growth medium affected the surface charge and colonial types of *N. gonorrhoeae* (27, 28). A very early report noted characteristic colonial types that could be isolated from patients suffering from acute or chronic forms of gonococcal infections (41). More recently, gonococci have been classified on the basis of colony edge morphology (19, 22) as well as opacity and color (39). Colony types T1 and T2 possess a fundamental virulence characteristic(s) not found in types T3 and T4 (5, 19–21). Moreover, transparent colonies isolated from women around the time of menses were both trypsin resistant and more virulent than the opaque colonies isolated from urethras of males or cervical cultures from women near the midpoint of their menstrual cycle (39).

Magnusson et al. (27) concluded that the gonococcal cell surface was more hydrophobic and slightly negatively charged at pH 7.2, whereas the same cells grown at pH 6.0 in an iron deficient medium lost the negative cell surface charge completely (28). On the other hand, cells grown at low pH in an iron-sufficient medium exhibited a negatively charged surface (27). Iron limitation in gonococci resulted in a change in the outer membrane protein profile (31). A detailed discussion of the effects of environment on bacterial surface composition has been presented by Ellwood and Tempest (10).

Heavy nasopharyngeal carriage of meningococci is often associated with inflammation (nasopharyngitis) (14). Since the inflammatory process characteristically involves a decrease in the pH as well as an increased excretion of the iron scavenger apolactoferrin from polymorphonuclear leukocytes (6, 7, 15, 40), the results we have reported here may simulate those under in vivo conditions.

It seems reasonable to assume that proteins exposed on the microbial cell surface interact directly with the environment or defense mechanisms in the host. Any changes in these proteins could affect this interaction to the advantage or disadvantage of the microorganism. In our experiments here, the sodium dodecyl sulfate-polyacrylamide gel electrophoresis outer membrane protein profiles of meningococci grown under iron-limiting conditions (pH 6.6 or pH 7.2) were similar to those of meningococci grown at pH 6.6 in sufficient iron in that protein III (molecular weight, 69,000) became a major protein. When cells at neutral pH had sufficient iron, this high-molecular-weight outer membrane protein (protein III) was no longer a major species. The appearance of this protein in itself cannot account for the increased virulence seen in growth under iron limitation or at low pH.

Only a few laboratories (18, 38, 43) have investigated the direct effects of in vitro growth conditions on the relative virulence of bacteria in an animal model. Strains of *Escherichia coli* harboring plasmid colV, which codes for an efficient iron sequestering system, are virulent, whereas non-colicinogenic strains are avirulent in a variety of laboratory animals (38, 43).

In the serum of the host, the major source of iron available is the iron-transport protein transferrin. Archibald and DeVoe (2) have reported that meningococci are capable of growth in a defined medium with human iron transferrin as the sole iron source. Holbein (17) has very recently shown that prolonged infection by meningococci leading to death in mice can be achieved only when iron levels on serum transferrin are maintained. Recent work by Simonson, Brener, and DeVoe (unpublished data) has shown that the iron from human transferrin is removed at the surface of the meningococcus in the absence of a soluble chelator. It appears that a rather sophisticated mechanism is present in the outer membrane of this organism for the uptake of iron from this serum protein. Whether this system is directly related to the increased virulence of iron-starved meningococci remains speculative.

When *N. meningitidis* was grown in an iron-limited medium at low pH, fewer than 10 bacteria killed a mouse. That virulence was so greatly enhanced by the combination of these two parameters suggests that iron acquisition from the host was only one (6, 42) factor among those which determine the virulence of this organism. It is conceivable that low pH and iron limitation mimic the in vivo conditions of the infected abdominal cavity in mice. If this were so, then meningococci already adapted to such environmental pressures would have a distinct

advantage over conventionally grown cells during the mouse challenge. Whether the manipulation of virulence in the meningococcus, as we describe here, is related to a more efficient iron acquisition mechanism remains an exciting possibility.

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