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Morphological Aberrations of Nutritionally Deficient Streptococci: Association with Pyridoxal (Vitamin B_6) Concentration and Potential Role in Antibiotic Resistance

RICHARD B. CLARK,[1]* RONALD E. GORDON,[2] EDWARD J. BOTTONE,[1] AND MARIA REITANO[1]

Departments of Microbiology[1] and Pathology,[2] The Mount Sinai Hospital and Mount Sinai School of Medicine, New York, New York 10029

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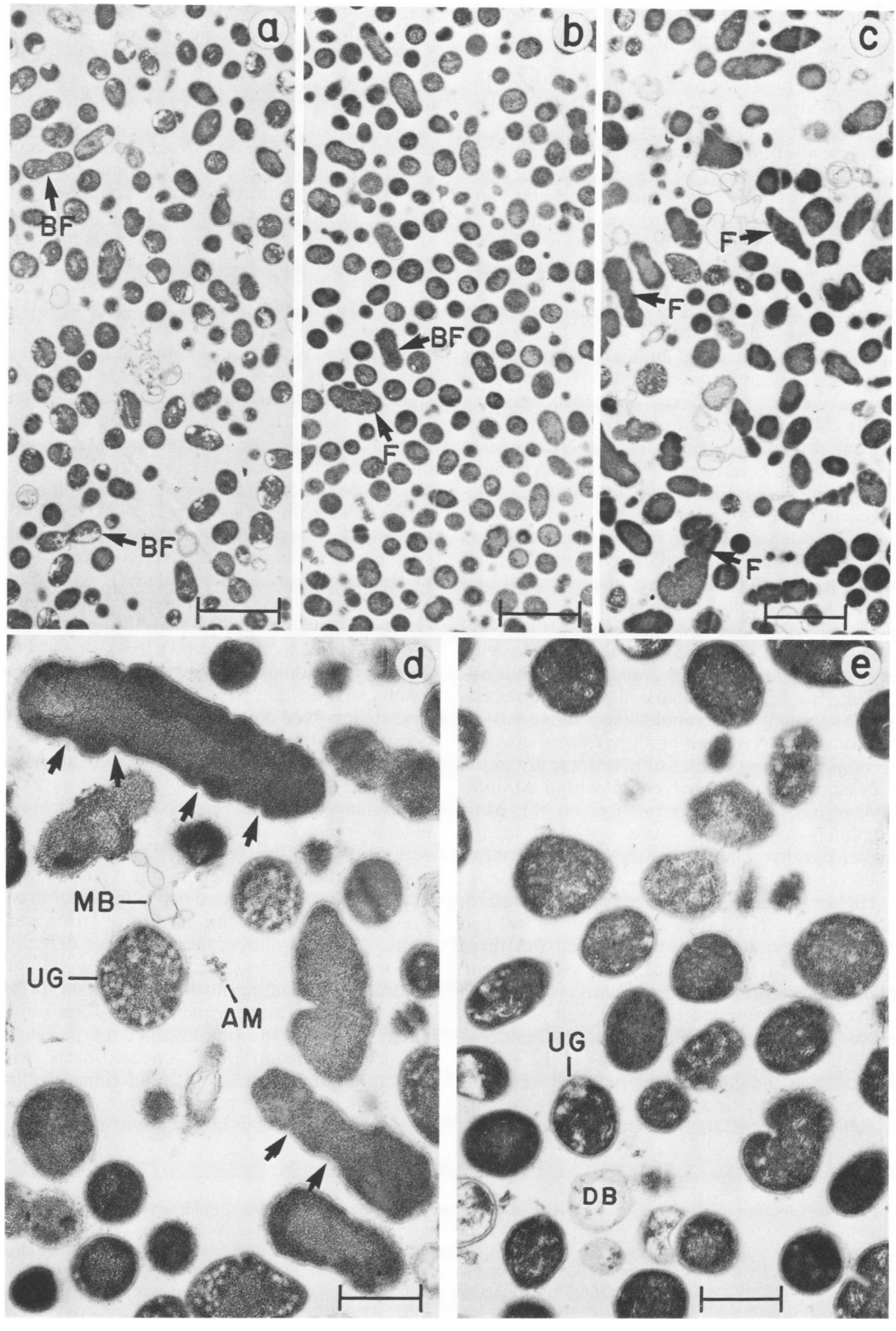
A strain of a nutritionally deficient streptococcus was shown to undergo morphological aberrations according to pyridoxal concentrations in the growth medium. Filamentous rod-shaped cells, observed by electron microscopy, predominated in the presence of decreasing concentrations. Multiple invaginations in the outer cell wall suggested inhibition of binary fission. Penicillin antimicrobial studies performed in the presence of similar pyridoxal concentrations indicated a relationship between filamentous forms and penicillin susceptibility.

Nutritionally deficient streptococci (NDS) have been reported as frequent causes of endocarditis and bacteremia in humans (6, 7). Growth of these organisms on routine laboratory medium is dependent upon the addition of exogenously added metabolites, including cysteine, thiol compounds, and activated forms of vitamin B_6 (pyridoxal and pyridoxamine) (4, 6). NDS will grow as satelliting colonies around host colonies of bacterial species elaborating the essential metabolite(s) (6). Gram-stained smears of the satellite colonies in close proximity to the host colony reveal typical gram-positive cocci in pairs and short chains; however, organisms smeared from the outer edge of the zone of satellitism are gram variable and pleomorphic, with globular and filamentous forms (5, 6). Factors accounting for the morphological aberrations noted among cells distal to the central colony may include diminution of essential compounds necessary for cell wall formation. Previous workers (2, 3) have demonstrated that pleomorphism of NDS is media dependent. In the present study, we show through light and electron microscopy that the noted morphological diversity characterizing NDS is conditional upon the pyridoxal concentration.

Five NDS isolates were recovered from blood culture specimens at The Mount Sinai Hospital during a 9-month period; one of these strains was selected and used throughout the present studies. This strain grew on 5% sheep blood agar (BBL Microbiology Systems, Cockeysville, Md.) as satelliting colonies around *Staphylococcus aureus*. The isolate was maintained on Todd-Hewitt agar (Todd Hewitt broth; Oxoid, London, England, plus 2% agar supplemented with 100 μg of pyridoxal HCl per ml; Sigma Chemical Co., St. Louis, Mo.). To assess quantitatively the morphological aberrations induced by pyridoxal, the NDS isolate was suspended in saline (approximately 10^7 CFU/ml), after which 0.1-ml samples were added separately to eight test tubes containing 19.9 ml of Todd-Hewitt broth supplemented with 10-fold decrements of pyridoxal ranging from 10,000 through 0.01 μg/ml. Unsupplemented Todd-Hewitt broth served as a negative control. After overnight incubation at 37°C, the cultures were inspected visually for turbidity, and samples were examined by light microscopy after Gram staining. Each tube showing visual turbidity was centrifuged at 1,000 × *g* for 10 min to pellet the organisms, after which the supernatant was discarded and the pellet was immediately fixed with 10 ml of 3% glutaraldehyde in a 0.2 M sodium cacodylate buffer (pH 7.4). The pellet was treated for 1 h with 1% osmium tetroxide buffer with sodium cacodylate and was dehydrated in graded steps of ethanol through propylene oxide and embedded in Epon. Epon sections (1 μm each) were cut on a LKB ultramicrotome and stained with toluidine blue, and smaller representative areas were chosen for thin sections. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a JEM 100 CX transmission electron microscope.

Growth of the NDS isolate occurred in Todd-Hewitt broth supplemented with pyridoxal ranging from 1.0 to 10,000 μg/ml, but not in the presence of 0.1 and 0.01 μg/ml or in unsupplemented Todd-Hewitt broth. Gram-stained smears prepared from cells grown in 1.0 and 10 μg of pyridoxal per ml showed gram-variable,



pleomorphic, globular coccobacilli in short chains. At higher pyridoxal concentrations, characteristic streptococcal morphology and staining intensity were observed.

Electron photomicrographs revealed that the ultrastructural morphology of the NDS isolate differed according to pyridoxal concentration. In the presence of descending pyridoxal concentrations, the morphology of the organisms became successively more filamentous, rod-like, and globular, and less coccal to coccobacillary. At the highest pyridoxal concentration (10,000 μg/ml, Fig. 1a), only streptococcal morphology consisting of cocci and coccobacilli (size range 0.6 to 1.2 μm) was observed. Filamentous or rod-like forms (size range 0.7 to 1.3 μm) were observed in increasing numbers at the lower pyridoxal concentrations of 100 to 10 μg/ml (Fig. 1b); however, the coccal and coccobacillary forms still predominated. At 1.0 μg of pyridoxal per ml, the ultrastructure of the NDS isolate consisted of a heterogeneous population of morphological forms (Fig. 1c), including cocci, coccobacilli, and filamentous rod-like bacterial cells. The length of the filamentous cells was significantly increased and varied from 1.4 to 4.4 μm, whereas the size range of the coccal and coccobacillary cells remained constant (size 0.7 to 1.2 μm). Close examination of the NDS isolate (Fig. 1a, b, and c) revealed the presence of intracellular unstained granules and cells appearing to undergo binary fission (dumbbell-shaped forms). The granules (size range 0.04 to 0.4 μm) were observed at all pyridoxal concentrations and were similar ultrastructurally to the polysaccharide granules noted within *Streptococcus mitis* cells by Berman et al. (1). Most dumbbell-shaped cells were observed at the higher pyridoxal concentrations and were infrequently seen at 1.0 μg/ml.

Higher magnification electron photomicrographs of the NDS isolate grown in 10,000 and 1.0 μg of pyridoxal per ml showed the three morphological forms (cocci, coccobacilli, and filamentous forms) to be relatively electron dense and surrounded by a cell wall and cytoplasmic membrane profile typical for streptococci (Fig. 1d and e). No differences in the size or thickness of the membrane structures were noted between the morphological forms at any pyridoxal concentration. Multiple invaginations were present in the outer cell walls of the filamentous forms (Fig. 1d). Morphologically, it appears that these bacterial cells initiated binary fission by forming invaginations but were unable to complete the formation of cross walls required for separation of the two daughter cells. As a result, the rod-like cells continued to increase in length. The fine structure of the coccal cells (Fig. 1d and e) appeared similar at both pyridoxal concentrations and contained unstained granules. Also seen were membrane structures with little or no internal electron density. The larger membrane structures (0.5 to 1.2 μm) may be degenerating bacteria, and the smaller structures (0.1 to 0.3 μm) could be membrane blebs pinched off from the outer bacterial cell wall. Amorphous, granular, dense material was observed in the extracellular spaces or bound to the outer cell walls of many bacterial cells. The degenerating bacterial cells, membrane blebs, and amorphous material were observed at all pyridoxal concentrations.

The observations described above indicate a direct relationship between pyridoxal concentration and the morphological presentation of NDS. At the lowest concentration supporting growth, filamentous forms predominated. This morphological aberration was analogous to that noted in Gram-stained smears of satelliting colonies growing distal to the central streak of the host species providing essential nutrient(s). These results suggest that pyridoxal is a requisite metabolite involved directly or indirectly with NDS cell wall synthesis (specifically, cross wall synthesis during binary fission). The clinical significance of these forms is not clear at present. Preliminary data from this laboratory indicate an inverse relationship between the bactericidal action of penicillin against NDS and pyridoxal concentrations contained in the test medium. Penicillin bactericidal activity is maximal in the presence of decreasing pyridoxal concentrations. As noted above, incomplete or defective cell wall synthesis at the lower pyridoxal concentrations, resulting in the filamentous forms, may actually enhance penicillin entry and subsequent activity. In the presence of increased pyridoxal concentrations, coccal forms with structurally intact cell walls predominate which may confer increased resistance (permeability) to penicillin. How these observations relate to the

FIG. 1. Electron micrographs of the NDS isolate grown in Todd-Hewitt broth supplemented with 10,000 (a), 100 (b), and 1.0 (c) μg of pyridoxal per ml. F indicates the presence of the filamentous forms. Bacterial cells undergoing binary fission are labeled BF. UG indicates bacterial cells containing unstained granules. (d and e) High magnifications of strain A grown in Todd-Hewitt broth supplemented with 1 (d) and 10,000 (e) μg of pyridoxal per ml. Arrows indicate the presence of multiple invaginations in the filamentous cells. Degenerating bacteria (DB) are present, together with intracellular unstained granules (UG), membrane blebs (MB), and amorphous granular material. Bar = 2 μm in a, b, and c; 0.5 μm in d and e.

utilization of penicillin to treat patients with NDS infections, especially endocarditis, remains to be evaluated.

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