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Virulence of *Nocardia asteroides* During Its Growth Cycle

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Cells of *Nocardia asteroides* undergo structural and chemical changes, especially in the cell wall, during growth in brain heart infusion broth. Experiments were devised to determine whether these changes affected the virulence of *Nocardia* for mice. It took, on the average, 1,380 times the number of colony-forming units at the stationary phase to achieve the same mortality induced by the log-phase cells. Cells in either the lag phase or early stationary phase of growth were intermediate in the numbers of colony-forming units required to kill mice. Dry-weight determinations at different stages of growth demonstrated that the log-phase organisms were approximately 10 times heavier than stationary-phase cells. Thus, on the basis of dry-weight (micrograms) values, the average colony-forming unit of log phase is approximately 130 times more virulent than in stationary-phase cultures. Therefore, the stage of growth affects greatly the virulence of *N. asteroides.*

Filamentous cells of *Nocardia* develop from coccoid organisms. These filaments fragment to form rods and cocci that repeat the cycle. It has been shown that ultrastructural and biochemical alterations occur within the cell envelope during this growth process (3, 5). Based on these observations, it was suggested that there might be corresponding changes in the host-parasite interaction. There have been numerous investigations concerning the virulence of *Nocardia* for mice. Many of these reports have had contradictory interpretations (1, 4, 6-21). Most of these studies used nocardial cell pellets or "crude" suspensions grown on various media for from 1 to several weeks (7-11, 13-15, 21). Frequently, the suspension was given to the animal with some form of "adjuvant" (e.g., hog gastric mucin, oil [1, 2, 6, 9, 11-13, 21]). Very little attention has been given to the effect of culture age on the possible virulence of strains of *Nocardia* (1, 2, 6-21). We found that by using a carefully prepared, homogeneous suspension of organisms separated by differential centrifugation followed by intravenous injection into mice, the relative virulence of several strains of *Nocardia asteroides* could be determined reproducibly (4). Thus, we had a system whereby we could study in more detail the effect specific factors such as culture age had on nocardial virulence.

MATERIALS AND METHODS

Microorganism. *N. asteroides* GUH-2 was isolated from a fatal human infection at Georgetown University Hospital, Washington, D.C. The patient had had a renal transplant, and the organism was isolated from the kidney. The grey clone was used during this study (4).

Growth studies. As indicated previously (3, 4), brain heart infusion (BHI, Difco Laboratories, Detroit, Mich.) broth supported the growth of *N. asteroides* in a dispersed and uniform suspension that allowed us to evaluate the various stages of development.

N. asteroides GUH-2 was isolated from the kidneys of mice 1 week after infection by homogenizing the kidneys in sterile BHI broth and transferring this suspension to BHI agar plates. The plates were incubated for 3 days at 34°C, and individual colonies were transferred to 50 ml of fresh BHI broth in a 250-ml Erlenmeyer flask that served as the starter culture for the growth experiment as previously described (3, 4). To study the growth and to determine the effect of culture age on virulence, flasks (250-ml Erlenmeyer containing 50 ml of BHI broth) were inoculated with 0.05 ml of the starter culture (72 h, and optical density [OD] of 5.5). The same culture used for the 1-week stationary-phase cells was used also to prepare the 3-day, 16-, and 5-h cultures. Thus, the properties of cultures from the four time periods were determined after using the same starter culture. The growth was quantitated by OD at 580 nm with a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.).

Determination of LD_{50} at different stages of nocardial growth. After 5 h (lag), 16 h (log), 3 days (early stationary), and 1 week (stationary) of incubation, the organisms were separated by differential centrifugation as previously described (4). The cells were resuspended in sterile saline (0.85% NaCl), and the concentration was adjusted so that 1.0 ml contained approximately 10^8 organisms. Ten-fold serial dilutions

in saline were made by using this initial suspension, and 10 mice in each group received 0.1-ml intravenous injections of the appropriate dilutions in the tail. At the same time, 5 ml of the highest concentration of cells were added to five clean, preweighed tubes and dried overnight at 110°C. Five tubes were also prepared by using saline without organisms. All dried tubes were weighed by using a Sartorius analytical balance. The blank, control tube and saline weights were subtracted from the samples containing organisms. The difference was taken as the dry weight of the organisms present in the sample. At the same time, samples were quantitated by plate counts of viable colony-forming units (CFU). Thus, the dry weight of the sample was divided by the numbers of organisms to give the mean dry weight per CFU. All values represented the mean of five preparations. Therefore, we determined both the number of CFU and the dry weight necessary to result in a 50% lethal dose (LD_{50}) during different stages of growth. The LD_{50} was determined by the standard methods of Reed and Muench (4). Each experiment (using at least 120 mice per determination) was repeated at least three times on three different preparations of *N. asteroides* GUH-2.

Animals. Female Swiss Webster mice, 4 weeks of age and averaging 18 to 20 g in weight, were obtained from Simonsen's (Gilroy, Calif.), and used throughout this study.

Electron microscopy. At 5, 16, 72, and 168 h, cell suspensions were fixed in 3.0% glutaraldehyde in Kellenberger buffer (pH 6.5), postfixed in OsO_4, and embedded in Maraglas as described previously (2, 3). Sections were cut by use of a diamond knife on a MT-2 Porter Blum ultramicrotome. Sections were collected and stained with lead citrate as described (2, 3). The sections were photographed through an AEI-801 electron microscope.

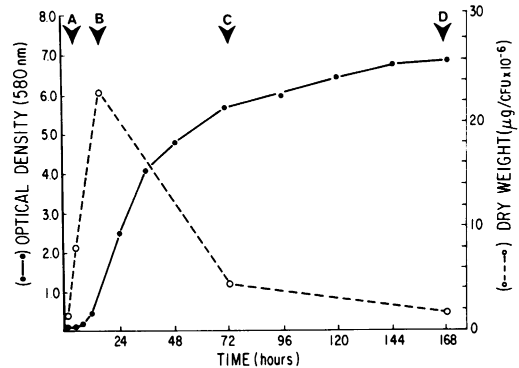

FIG. 1. *Growth curve of N. asteroides GUH-2 inoculated into BHI broth and incubated at 34°C and 150 rpm rotational agitation. Symbols: OD, ●——●; microgram dry-weight values per CFU, ○- - -○. Experimental samples were taken at (A) lag phase, 5 h; (B) log phase, 16 h; (C) early stationary phase, 72 h; and (D) late stationary phase, 168 h.*

RESULTS

The growth curve for *N. asteroides* GUH-2 in BHI broth was standardized as described above (Fig. 1). Dry-weight determinations of cell suspensions at each time period demonstrated that during lag phase (i.e., no increase in CFU per ml) there was a significant increase in cell weight that reached a maximum at about 16 h (early logarithmic phase) post-inoculation. By light microscopy, at 5 h (lag phase) the cells had begun to enlarge and elongate. At 16 h post-inoculation, the cell suspension was composed entirely of long, branching filaments with an average dry weight of 22×10^{-6} μg/CFU, or about 10 times heavier than the average CFU used for the inoculum (Fig. 1). These branching filaments continued to fragment and elongate during log-phase growth, but as the growth rate slowed, the average filament length and weight decreased. Thus, in stationary phase almost 100% of the cells were short rods and cocci with an average dry weight of 1.8×10^{-6} μg/CFU (Fig. 1).

Electron microscopy revealed corresponding changes in cell ultrastructure, with the cell wall being altered the most (Fig. 2). Log-phase cells possessed a thin trilayered cell wall (Fig. 2A). The outer layer is prominent and osmiophilic (Fig. 2a). This layer has been shown to be composed primarily of lipids and peptide amino acids with some carbohydrate (3). The inner layer of the wall is less dense than the outer layer, and it appears to be about the same thickness. The electron-lucent zone between the outer and inner layers is composed mostly of carbohydrate and lipid material, whereas the inner layer is peptidoglycan and carbohydrate (3). In sharp contrast, the stationary-phase cells have a greatly thickened cell wall (Fig. 2B). The outer layer becomes much less osmiophilic, and has correspondingly less lipid than the cell wall of log-phase organisms (3). The innermost layer has become prominent (Fig. 2b), and now the cell wall consists of mostly peptidoglycan and carbohydrate (3).

Groups of 10 mice were injected intravenously with cell suspensions, and the LD_{50} was determined for *N. asteroides* GUH-2 in (A) lag phase (5 h), (B) log phase (16 h), (C) early stationary phase (3 days), and (D) late stationary phase (1 week) of growth in BHI broth (Table 1). A total of 120 mice were used for each experiment, and this was repeated on three separate cell populations prepared several weeks apart to establish the reproducibility of the system. Table 1 shows that there is a dramatic difference in the viru-

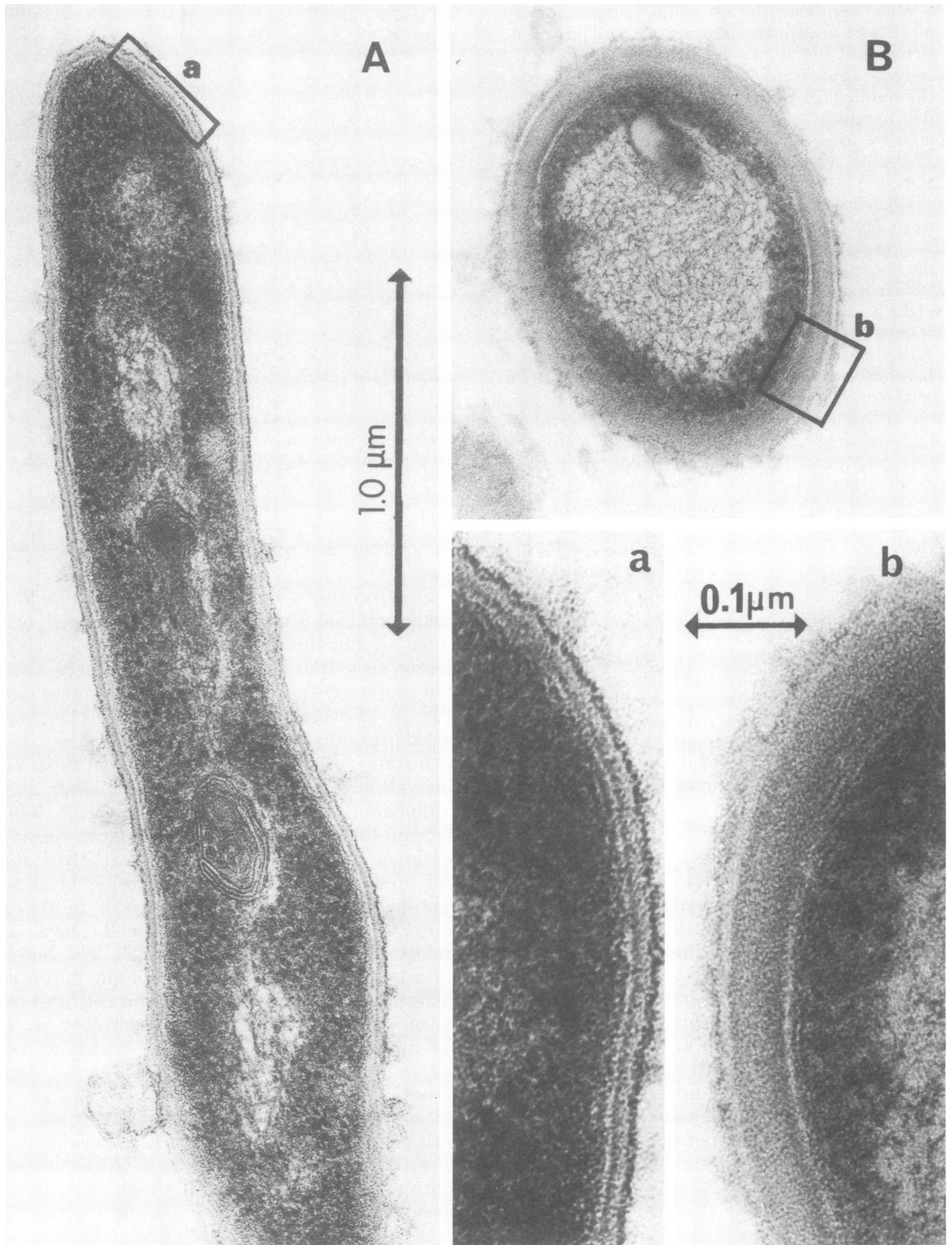

FIG. 2. *Electron micrographs of N. asteroides GUH-2 during its growth cycle (Fig. 1). (A) Thin section of log-phase cells (16 h) taken at (B) in Fig. 1. (a) High magnification of a portion of the cell wall from (A) showing cell wall structure. (B) Thin section of stationary-phase cells (168 h) taken at (D) in Fig. 1. (b) High magnification of a portion of the cell wall from (B) showing cell wall structure. Both (A) and (B) are the same magnification, and the bar represents 1.0 μm; (a) and (b) are magnified the same, and the bar represents 0.1 μm.*

TABLE 1. *Effect of culture age on the virulence of N. asteroides GUH-2 for mice*[a]

No. of organisms	Culture age (h)	No. of mice inoculated	Deaths after 4 weeks	% Deaths	Calculated LD_{50} (CFU)
A. 5 h					
4.3×10^6	5	10	10	100	
4.3×10^5	5	10	4	40	6.3×10^5
4.3×10^4	5	10	0	0	
B. 16 h					
3.0×10^5	16	10	10	100	
3.0×10^4	16	10	5	50	2.0×10^4
3.0×10^3	16	10	2	20	
C. 3 days					
2.5×10^7	72	10	10	100	
2.5×10^6	72	10	4	40	3.7×10^6
2.5×10^5	72	10	0	0	
D. 1 week					
2.1×10^8	168	10	10	100	
2.1×10^7	168	10	1	10	7.5×10^7
2.1×10^6	168	10	0	0	

[a] These data come from a single representative experiment. The experiment was repeated three times, and the average values are shown in Table 2.

TABLE 2. *Mean LD_{50} values of N. asteroides GUH-2 injected intravenously into mice*[a]

A. Mean LD_{50} at 5 h (lag)	B. Mean LD_{50} at 16 h (log)	C. Mean LD_{50} at 3 day (stationary)	D. Mean LD_{50} at 1 week (stationary)
3.9×10^5 CFU/mouse (3.1 μg/mouse)[b]	3.4×10^4 CFU/mouse (0.75 μg/mouse)	2.9×10^6 CFU/mouse (11.6 μg/mouse)	4.7×10^7 CFU/mouse (94.0 μg/mouse)

[a] The mean value of three separate experimental determinations.
[b] Microgram dry-weight determinations.

lence of the organism depending upon the stage of growth. It took, on the average, 1,380 times the number of CFU at the stationary phase (1-week culture) to achieve the same mortality induced by log-phase cells (16-h culture) when all other experimental factors were constant and identical (Table 2). Furthermore, because the log-phase cells are approximately 10 times heavier (Fig. 1) than the 1-week stationary-phase organisms, it requires 130 times as much nocardial cell mass in stationary phase to bring about the same mortality rate achieved with the log-phase cells. In one experiment (Table 1) as few as 3,000 CFU of log-phase (16-h) cells killed 2 out of 10 mice within 1 month postinfection. Necropsies revealed massive kidney infections (characteristic of this strain [4]), with the organism being isolated in pure culture from the infected kidneys. The remaining mice were sacrificed at 1 month postinfection. All of these had lesions in the kidneys, and *N. asteroides* were readily isolated from the infected tissues (Table 1). In contrast, no mice died after receiving 2.1 $\times 10^6$ CFU of the 1-week (late stationary-phase) culture. Further, *N. asteroides* could not be isolated from the kidneys and the mice showed no signs of infection 1 month postinfection. Whereas the infectious dose appeared to be affected by the stage of bacterial growth, the target organ specificity for the kidneys demonstrated by this organism was not altered as a consequence of culture age.

DISCUSSION

These data clearly establish that to determine the relative virulence of a strain of *Nocardia* all conditions of the experimental design must be controlled. The growth conditions and culture age must be defined and the route of infection must be indicated. There have been a large number of reports in the literature concerning the relative virulence of several strains of *Nocardia*. There have even been statements that *N. asteroides* cannot infect an uncompromised host. Some individuals claim that *N. braziliensis* is more virulent than *N. asteroides,* whereas others claim the opposite to be true (1, 2, 6–21).

Unfortunately, the data presented by these investigators do not take into account the relative growth differences among strains (i.e., some strains grow much more rapidly in a given medium than others [Beaman, unpublished data]), the type of medium being used for growth, or the route of inoculation (there is a profound difference in mouse susceptibility to the same nocardial inoculum depending entirely upon route of inoculation [Beaman, unpublished data]).

We have found that alterations in methods of inoculation of the flasks (i.e., 1 ml of starter culture per 50 ml of broth versus 0.05 ml of starter culture per 50 ml of broth) significantly altered the growth curve characteristics of the organism. This resulted in a change in the virulence of the organism. For example, a sample of *N. asteroides* GUH-2 taken from a 3-day starter culture (obtained by inoculating an infected mouse kidney into BHI broth) incubated at 34°C with 150 rpm rotational agitation has an OD (580 nm) of 5.5 ± 0.2. If 1 ml of this starter culture is then inoculated into 50 ml of fresh BHI in a 250-ml flask the starting OD is 0.1, and after 16 h of incubation the OD is approximately 2.5. Phase-contrast microscopy of this preparation shows that the cells are branching filaments, and the LD_{50} for mice (intravenous infection) is 8×10^5 CFU (B. L. Beaman and S. Maslan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, B46, p. 23). If on the other hand, 0.05 ml of the starter culture is added to 50 ml of BHI broth as described above, the resultant OD is not measurable (approaches 0.01 at 580 nm) and at 16 h of incubation the OD of this preparation is only 1.5. Phase-contrast microscopy of this preparation showed that the cells are branching filaments indistinguishable (by light microscopy) from those seen in the series described above. However, the LD_{50} for mice (intravenous injection) was 2.0×10^4 CFU (Table 1). Thus, there was a marked difference in the virulence of cells of *N. asteroides* GUH-2 grown for 16 h that depended upon how the experiment was started. After 1 week of incubation (stationary phase), there is little or no difference in virulence between these two cultures. The organisms in the first flask had an LD_{50} for mice of 8×10^7 CFU (B. L. Beaman and S. Maslan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, B46, p. 23), whereas organisms in the second flask had an LD_{50} of 3.7×10^7 CFU. Interestingly, the final OD in both flasks after a 1-week incubation was 7.0. Similar results were obtained in replicate experiments. Therefore, the culture age affects virulence of *N. asteroides* GUH-2 regardless of how the experiment was performed, but the way in which the cultures were initially started affects the relative degree of this difference.

These data suggest that the chemical changes that occur in the cell wall during the growth cycle of *N. asteroides* play a significant role in the host-parasite interaction. Therefore, an analysis of the individual components within the walls of the organisms during different stages of growth should further permit chemical definition of these components and their role in nocardial virulence and host response.

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