The Influence of Growth Medium on Axenic Cultivation of Virulent and Avirulent *Acanthamoeba*[1] (37346)

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Primary amebic meningoencephalitis is recognized as a human disease attributable to infection with strains of the free-living amebae, *Naegleria* or *Acanthamoeba* (1–5). While only *Naegleria* has been verified in a few of the human cases by cultural isolation, the ability of *Acanthamoeba* to produce encephalitis in rodents and primates (4) indicates that strains of both genera must be considered facultative human pathogens.

In recent years, a variety of reports have appeared that have considered the taxonomy, biological characteristics, and virulence of *Acanthamoeba* strains (3, 4, 6). Nevertheless, only limited information is available on the growth of pathogenic *Acanthamoeba* in axenic culture. This report deals specifically with a description of the growth parameters of two pathogenic strains of *Acanthamoeba,* A-1 (acutely pathogenic) and HN-3 (chronically pathogenic) (4) in an axenic, aerated suspension environment. A comparison is made of the growth of these strains to the growth of the nonpathogenic (Neff) strain of *Acanthamoeba* cultured under similar conditions. A subsequent report will deal with the effect of cultivation under the established optimal culture conditions on the virulency of the acutely pathogenic *Acanthamoeba.*

Materials and Methods. Strains of Acanthamoeba. Axenic cultures of *A. castellanii* (Neff) were obtained from Dr. R. J. Neff (Vanderbilt University). The strain was originally isolated from a soil sample at Pacific Grove, California (7). Axenic cultures of A-1 and HN-3, originally isolated from a monkey kidney tissue culture and a human nasopharynx, respectively (4), were supplied by Dr. C. G. Culbertson (Eli Lilly Laboratories).

Culture media. The Neff strain was cultured in an axenic medium described by Neff *et al.* (8). A modified Neff medium, containing serum and an increased concentration of proteose peptone, was used for growth of A-1 and HN-3. Other media components were the same as for the Neff strain. Dialyzed fetal calf serum (DFCS) (Gibco) was added under sterile conditions just before subculturing with a Swinnex filtering unit (Type HA, 0.45μ) to a final concentration of 1.0% in the culture medium.

Aerated suspension growth. Except for minor modifications, all three strains were cultured in an aerated suspension system as originally described by Neff *et al.* (8) in a cell culture area maintained at 29–31°. The major modification was the removal of the sampling ports from all set-ups. Sampling was carried out routinely with sterile disposable syringes and 20G-1½ inch needles inserted directly into the incoming flask tubing. All additions to the culture flasks were accomplished in a manner identical to that used for sampling. A more detailed description of the modifications with accompanying illustrations will be found in O'Dell and Stevens (9).

After autoclaving, suspension set-ups were stored at 4° and used within a week. Following use, flasks containing pathogenic species were killed by addition of a disinfectant and subsequently autoclaved. Flasks were washed and desiliconized according to

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Neff *et al.* (8).

Individual flow meters (Matheson-Cole and Bell) were used to monitor the aeration rate of incoming house air. The flow rate was 3 cfh/liter of medium for growth of Neff and HN-3 and 4 cfh/liter for A-1.

Subculturing and cell counting. Subculturing was carried out when cell cultures were in exponential growth. Fresh cultures were inoculated at a concentration of 1–2 $\times$ 10^3 cells/ml. All cell counts were performed using phase optics with a Neubaur-Bright Line Hemocytometer. Pathogenic strains were fixed prior to counting by the addition of formalin (1.0%).

Results. As seen in Fig. 1, the Neff strain grew with a generation time of 7–8 hr under the described conditions. The cells grew to an exponential density of 2.5–3.0 $\times$ 10^6 cells/ml and then entered a period of growth deceleration during which the growth rate decreased approximately 4-fold (Fig. 1). After 25–35 hr post-exponential growth, stationary population densities were obtained. By 7–10 hr into stationary phase, cysts began to appear in the cultures and increased to 100% over the ensuing 24 hr. A more detailed description of the kinetics of encystment of *A. castellani* (Neff) in optimal growth cultures will be found in Stevens and Pachler (10).

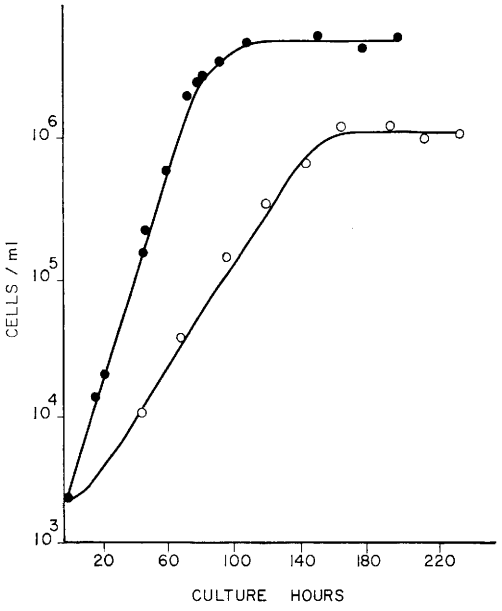

FIG. 1. Growth kinetics of the Neff strain of *Acanthamoeba* in aerated, suspension culture in Neff medium (●—●); 4.0% proteose peptone Neff medium (4%-Neff) (○—○).

Throughout the period of exponential growth, the amebae were of uniform size, exhibited moderately vacuolated cytoplasm and contained a single active contractile vacuole. Multinucleates were rare in logarithmically growing cultures and increased to roughly 5–10% in stationary cultures.

Growth of HN-3 amebae in the aerated suspension system was attempted initially in trypticase–soy broth or Neff medium. The former medium had been used routinely for axenic cultivation of HN-3 (and A-1) in monolayer cultures by Culbertson (4). Although long periods were allowed for adaptation of the strain to suspension growth, neither of these media supported good growth of the cells. The cells grew slowly or not at all, were extremely variable in size, highly granulated and entered stationary phase at low population densities.

Subsequent attempts to modify the Neff medium led to the observation that increasing the proteose peptone concentration to 4.0% resulted in an enhancement of growth of HN-3. This modified medium, designated 4.0%-Neff medium, provided a generation time of 22–24 hr and higher exponential densities (Fig. 2) than obtained with either the trypticase–soy broth or unmodified Neff medium. However, after repeated subculture, it was noted that the 4.0%-Neff medium failed to provide a consistent level of exponential growth or a reproducible growth rate.

Various workers have reported the use of serum for axenic cultivation of pathogenic amebae (11); Nelson (personal communication). For this reason, we further enriched the 4.0%-Neff medium with DFCS. With the first subculture into medium containing 1.0% DFCS, the HN-3 exhibited remarkably uniform and consistent growth. The cells attained stationary population densities between 1–2 $\times$ 10^6 cells/ml and grew with a generation time of 14–16 hr. The population doubling time stabilized at a constant 10–11 hr by the 20th subculture. The profile shown

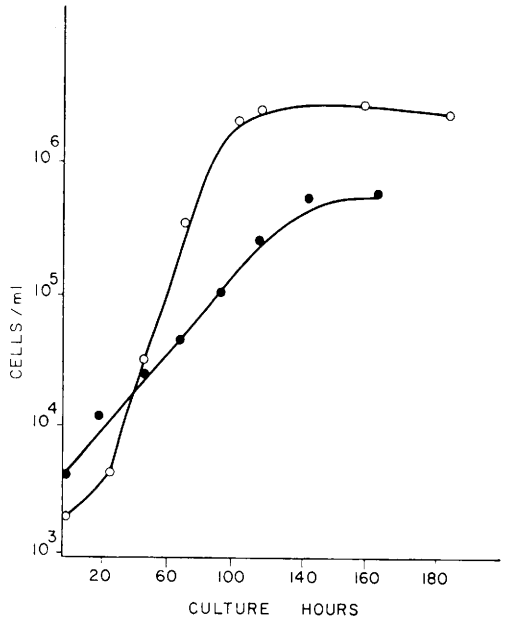

FIG. 2. Growth kinetics of the HN-3 strain of *Acanthamoeba* in aerated, suspension culture in 4%-Neff (●—●); 4%-Neff with 1.0% dialyzed fetal calf serum (4%-Neff-DFCS) (○—○).

in Fig. 2 is representative of the growth of HN-3 in the 4.0%-Neff-DFCS medium from the 20th through 84th subcultures (665 generations).

Attempts to further enhance the growth of HN-3 in the 4.0%-Neff-DFCS medium with additional serum (1.0%) added either in mid- or late exponential growth were unsuccessful. Higher concentrations of serum provided from the onset of growth may elicit a greater growth response; however, this was not examined since the increased protein concentration led to considerable foaming and loss of medium from the flask exhaust.

HN-3 trophozoites cultured in the 4.0%-Neff-DFCS medium were similar in appearance to Neff ameba grown in suspension. In unfixed samples from exponentially growing cultures, the amebae exhibited normal acanthapodia after being allowed to settle on the counting slide and a moderate to high amount of vacuolization. The cell size was slightly variable, which may be related to the greater percentage of multinucleated cells (10%) in the population during exponential growth. Encystment began in cultures approximately 15 hr postlogarithmic growth. Within 72 hr, greater than 90% encystment was obtained.

Due to our success with the serum-enriched Neff medium for the growth of HN-3, the highly virulent A-1 strain was inoculated from monolayer culture into the suspension system with this medium. With the initial subcultures, the cells grew with a generation time of 20 hr and to variable stationary densities; however, within 30 subcultures (200 generations), the A-1 cells were growing to consistent stationary densities and had a constant generation time of 8–9 hr (Fig. 3).

The striking difference between the growth of A-1 and HN-3 in the 4.0%-Neff-DFCS medium was the longer period of exponential growth and higher stationary densities attained by A-1. As noted in Fig. 3, A-1 *Acanthamoeba* grew logarithmically to 2.5–3.5 $\times$ 10^6 cells/ml and entered stationary phase at a population density of approximately 5 $\times$ 10^6 cells/ml. The stationary density remained constant for an extended period, *i.e.*, 85 hr-post-exponential growth, during which encystment was only rarely observed. When noted, the percentage of cysts

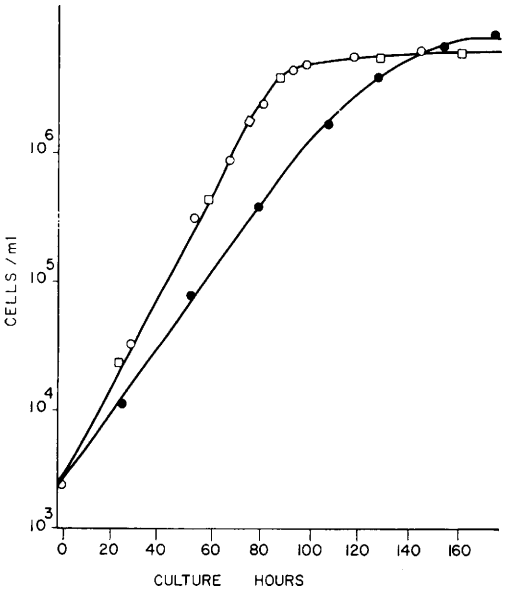

FIG. 3. Growth kinetics of the A-1 strain of *Acanthamoeba* in aerated, suspension culture in 4%-Neff-DFCS (□—□); 4%-Neff (○—○); Neff medium (●—●).

never exceeded 5.0% of the total cell population. Nevertheless, the cells had not lost the capacity for encystment; encystment readily occurred when A-1 cells were removed from axenic culture and inoculated into bacterized broth.

An unexpected finding was that the omission of serum from the 4.0%-Neff medium was without effect on the growth of A-1 amebae. Figure 3 demonstrates that the generation time and level of growth was equivalent in serum-containing or serum-free 4.0%-Neff medium. Subsequent to this finding, the A-1 strain was cultured routinely in the serum-free 4.0%-Neff medium and has exhibited the growth parameters shown in Fig. 3 for >100 sub-cultures (700 generations).

Since the A-1 strain appeared to be less fastidious than HN-3 in its nutritional requirements, we also attempted growth of the cells in the unmodified Neff medium. This medium supported stationary population densities slightly higher than those obtained with the 4.0%-Neff medium. However, the cells grew with a longer generation time and terminated exponential growth earlier than in the enriched (4.0%) Neff medium (Fig. 3).

The morphology of the A-1 amebae was not significantly different in any of the three media tested for growth. Generally, the cells were morphologically indistinguishable from the Neff and HN-3 strains. The cytoplasm was moderately vacuolated, granular and contained a single active contractile vacuole. Minimal size deviation was observed after adaptation, and multinucleates (< 2.0%) were rare in logarithmically growing cultures.

In view of the results obtained with the growth of A-1 and HN-3 in a modified Neff medium, a final comparison was made to determine if growth of the Neff strain could be stimulated either by increasing the concentration of proteose peptone or with the addition of serum. As seen in Fig. 1, Neff amebae exhibited poor growth in the presence of a higher concentration of proteose peptone. The growth rate was extended to 14–15 hr, and the cells terminated exponential growth at population densities below that attained in unmodified Neff medium. Although the addition of serum (1.0% DFCS) did not adversely affect growth, neither did it stimulate a greater growth response. The latter was true whether the Neff strain was grown continuously with the serum or whether serum was added in late exponential growth. Encystment of the Neff strain was unaffected in cultures containing serum.

Discussion. Since Culbertson initially reported that a strain of *Acanthamoeba* (A-1) was capable of causing severe meningoencephalitis after intransal instillation into mice and monkeys (12), considerable attention has been focused on the disease-producing potential of strains of *Acanthamoeba* and *Naegleria*. A significant number of clinical reports have appeared implicating either *Acanthamoeba* or *Naegleria* as the etiological agent in cases of human encephalitis (see Ref. (3) for review). These reports have been paralleled by more extensive analyses of the pathogenesis and chemotherapy of the disease in mice (3, 4). Nevertheless, the factors that enable the amebae to invade the mucosal membranes and to subsequently cause death of the host have not been defined. Such information may only be derived from molecular distinction of virulent and avirulent strains.

In order to facilitate comparative biochemical studies, we have developed culture conditions that provide optimal, axenic growth of pathogenic *Acanthamoeba*. Under the established conditions, the cells grew with constant generation times, to reproducible stationary densities and maintained the growth characteristics with repeated subcultivation. Although similar culture conditions were provided for the two strains of pathogenic *Acanthamoeba*, the growth responses of the cells could be easily distinguished. The greater growth response and lower probability of encystment of A-1 in comparison to the HN-3 strain observed in the present studies may relate to the reported differences in the ability of the two strains to produce acute or chronic brain lesions (4). A-1 produces massive hemorrhagic destruction which invariably results in the death of experimental mice. HN-3, however, produces only cerebral granulomata that contain cysts

and are often resolved without further pathology or death. Work is in progress to elicit factors which enable pathogenic amebae to penetrate mucosal membranes and proliferate in neural tissue.

Summary. The growth characteristics are described for two strains of pathogenic *Acanthamoeba* cultured in an axenic, peptone-based medium under aerated suspension conditions. The highly virulent A-1 amebae grew exponentially with a constant generation time of 8–9 hr to densities of 2.5–3.5 $\times$ 10^6 cells/ml. The HN-3 chronically virulent strain, whose growth medium was enriched with serum, exhibited a generation time of 10–11 hr and attained exponential densities of 1–2 $\times$ 10^6 cells/ml. Encystment readily occurred in stationary phase cultures of HN-3 cells, but not in A-1 cultures. A comparison was made of the growth parameters of the pathogenic *Acanthamoeba* to the nonpathogenic Neff strain cultured under similar conditions.

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