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Growth of *Haemophilus influenzae* in Simulated Blood Cultures Supplemented with Hemin and NAD

MICHAEL ARTMAN,* ELIZA DOMENECH, AND MATEI WEINER
Division of Microbiology and Immunology, Methodist Hospital, Brooklyn, New York 11215

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The mean generation time of *Haemophilus influenzae* in simulated blood cultures is 103 to 107 min. With 0.56 to 0.58 doublings per h, even large inocula of 256 cells per ml reach only 2×10^6 cells per ml and produce no visible evidence of growth after 24 h of incubation. Hemin and NAD added to simulated blood cultures triple the rate of growth of *H. influenzae*, so that even small inocula produce visible turbidity after overnight incubation. With a mean generation time of 36 min, a single cell of *H. influenzae* in simulated blood culture supplemented with hemin and NAD undergoes 30 doublings in 18 h, producing 2^{30} (1.07×10^9) cells and visible turbidity.

Prompt detection and identification of *Haemophilus influenzae* in blood cultures is of great importance in clinical microbiology. *H. influenzae* is the most frequent pediatric pathogen, and *Haemophilus* bacteremia is one of the most frequent causes of positive blood cultures in children 6 months to 5 years old (16, 17). An increase in *Haemophilus* bacteremia in older children and adults has been reported (7, 10, 13). At the same time *H. influenzae* is the microorganism that blood cultures most frequently fail to recover (3).

The detection of microorganisms in blood cultures depends primarily on the production of turbidity by the multiplying bacteria or on a positive growth index reading when the BACTEC radiometric system is used.

The current methods of growing *H. influenzae* in blood cultures are clearly not optimal. *H. influenzae* fails to produce turbidity after overnight incubation, and with low-density inocula, visual observation fails to detect the organism even after several days of incubation (4). The BACTEC radiometric system also fails to register positive growth index readings by 24 h in a large percentage of blood cultures from pediatric patients with high-grade *Haemophilus* bacteremia (12). Blind subcultures are, therefore, essential for the detection of *H. influenzae*, as these organisms are detected first only by subculture, never by macroscopic examination or Gram stain (4).

Recently it has been reported that counterimmunoelectrophoresis is capable of detecting *H. influenzae* type b antigen in macroscopically negative blood cultures with fewer than 10^5 cells per ml of medium (2).

In this communication, we report that the addition of hemin and NAD to simulated blood cultures enhances the growth rate of *H. influenzae* to such an extent that even small inocula produce visible turbidity after overnight incubation.

MATERIALS AND METHODS

Bacterial strains. Five recent isolates of *H. influenzae* type b from the microbiology laboratory of the Methodist Hospital and the following strains obtained from the American Type Culture Collection (Rockville, Md.) were used: *H. influenzae* ATCC 19418 and *H. influenzae* type b ATCC 9795, ATCC 10211, ATCC 31441, and ATCC 33533.

Culture media. Blood culture media used in this work consisted of 50 ml of brain heart infusion broth with sodium polyanethol sulfonate, *p*-aminobenzoic acid, and CO_2 (GIBCO Laboratories, Lawrence, Mass.). For the experiments, the media were supplemented with hemin, NAD, or hemin plus NAD, each at a concentration of 10 μg/ml of medium. Unsupplemented media served as controls. Blood culture bottles with and without the growth factors were stored at room temperature for 4 to 6 weeks without any sign of deterioration.

Hemin and NAD stock solutions. Hemin and NAD (β-NAD) were obtained from Sigma Chemical Co., St. Louis, Mo. Hemin and NAD stock solutions were prepared as previously described (1).

Inocula and viable counts. The bacteria were propagated on chocolate agar plates for 19 to 24 h. Five to 10 colonies were suspended in tryptic soy broth to give visible turbidity. Tenfold serial dilutions through 10^{-9} were made. The total viable count was made by spreading 0.1 ml of several dilutions on the surface of chocolate agar plates. To check for possible contamination, 0.1 ml of the undiluted bacterial suspensions was spread on the surface of sheep blood agar plates.

Simulated blood cultures. Blood was collected from

TABLE 1. Growth of *H. influenzae* from single-cell inocula

H. influenzae growth medium	Initial no. of bacteria (a_0)	Final no. of bacteria (a_n)	No. of generations (N)[a]	Incubation time (h)	MGT (min)	Doublings per h[b]
Brain heart infusion broth	1	1	0	24		0
Simulated blood culture	1	1.6×10^4	14	24	103	0.58
Chocolate agar	1	2.7×10^8	28	19	41	1.46
Brain heart infusion broth + hemin + NAD	1	3.3×10^7	25	15	36	1.66

[a] N when $a_0 = 1$ was calculated from the equation $N = \log_{10} a_n / \log_{10} 2$.
[b] Determined as the reciprocal of the MGT times 60; the exponential growth rate constant.

healthy donors under aseptic conditions. Blood was drawn into a syringe and inoculated on a 10% (vol/vol) basis into each of two blood culture bottles, one with and the other without hemin and NAD. In some experiments, two additional bottles were used, one with NAD only and the other with hemin only. One milliliter of bacterial suspensions from dilutions containing the desired number of cells was injected into all blood culture bottles. The bottles were vented and incubated at 36°C. Immediately after inoculation and at various time intervals, 1-ml samples were withdrawn from all blood culture bottles, and the total viable count was determined as described above.

Colony size and single-cell inocula. Well-isolated colonies were obtained on chocolate agar plates streaked with dilute bacterial suspensions containing 20 to 100 CFU/ml and incubated at 36°C for exactly 19 h. Each colony was touched with the tip of a sterile wood applicator or with a bacteriological loop, lifted off the agar, and transferred to 5 ml of tryptic soy broth. The bacteria were uniformly suspended, and a viable count was performed as described. For single-cell inocula the "single burst" method of Ellis and Delbrück was used (8). In this method, study of individual cells was made possible by the application of the Poisson equation to predict the distribution of bacteria in small samples of highly dilute suspensions.

RESULTS

Single-cell inocula. The results of a representative experiment on the growth of *H. influenzae* from single-cell inocula are presented in Table 1. Unsupplemented blood culture medium did not support the growth of *H. influenzae*. With the addition of human blood it became a simulated blood culture. In this medium, *H. influenzae* multiplied and reached 1.6×10^4 cells after 24 h of growth at 36°C. This involved 14 doublings with a mean generation time (MGT) of 103 min and 0.58 doublings per h.

These data are in agreement with those of Painter and Isenberg (15), who found that inocula of 290 cells of *H. influenzae* multiplied in simulated blood cultures over a period of 24 h, reaching greater than 10^6 cells, i.e., approximately 13 to 14 doublings with an MGT of 103 to 110 min.

The data presented in Table 1 also show that simulated blood cultures provide suboptimal conditions for the multiplication of *H. influenzae* as compared to those provided by chocolate agar media. Thus, a single cell incubated for 19 h on the surface of a chocolate agar plate gave rise to a colony consisting of 2.7×10^8 cells, which involved 28 doublings with an MGT of 41 min. Determination of the cell number of a large number of colonies revealed that they varied in size with the number of cells, ranging from 3.35×10^7 (MGT, 42 min) to 3.5×10^8 (MGT, 40 min).

It should be recalled that the rate of growth of bacteria on solid media is not optimal, because different parts of a colony differ in the oxygen availability from the air above the colony and in the availability of nutrients from the agar below (6).

The highest rate of growth of *H. influenzae* was observed when the bacteria were growing in brain heart infusion broth supplemented with hemin and NAD. Under these conditions, a single cell gave rise to progeny of 3.3×10^7 cells in 15 h. This involved 25 generations and an MGT of 36 min (1.66 doublings per h).

Simulated blood cultures. Population curves of *H. influenzae* growing in simulated blood cultures with and without hemin and NAD are shown in Fig. 1. The curves were plotted semilogarithmically to the base 2 of the number of cells. With this plot, each unit on the ordinate represents a doubling in population, so that a mere inspection of the curve permits one to determine the number of bacteria, the number of generations, and the generation time at any phase of growth.

In the absence of hemin and NAD, the cells entered a lag phase of 5-h duration immediately after incoulation. Thereafter, they began to divide. A decline in the growth rate was noted after 8 h, as evidenced by the change in slope. The bacteria multiplied from the initial 2^8 (256) cells per ml to 2^{21} (2.09×10^6) cells per ml after 24 h of incubation. This involved 13 doublings with 0.58 doublings per h, on the average.

In the presence of hemin and NAD, the cells entered the exponential phase of growth immediately after inoculation. When the inoculum

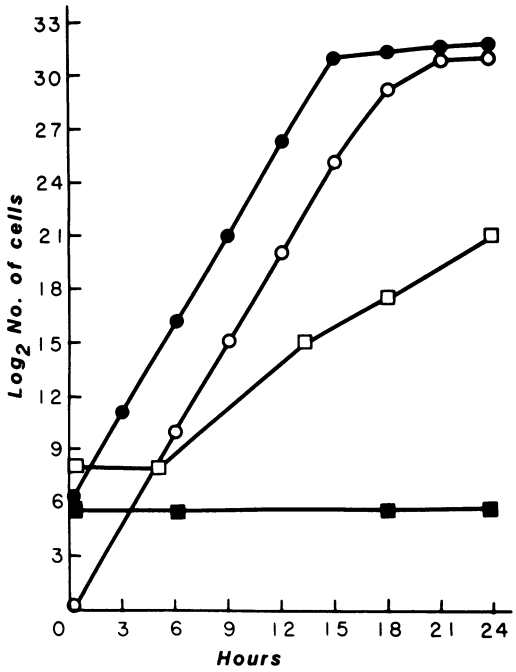

FIG. 1. Growth of *H. influenzae* type b in simulated blood cultures with and without hemin and NAD. Symbols: ○ and ●, blood cultures supplemented with hemin and NAD; □, blood culture without hemin and NAD (and also blood culture supplemented with hemin alone); ■, brain heart infusion broth without hemin and NAD.

consisted of $2^{6.3}$ (80) cells per ml, the bacteria underwent 25 doublings in 15 h, 1.7 doublings per h and an MGT of 36 min, reaching 2^{31} (2.15×10^9) cells per ml and producing visible turbidity. Thereafter, stationary phase ensued with no detectable change in the viable count for the duration of the experiment. When the inoculum consisted of 1 cell per ml, the MGT remained unchanged and the bacteria reached their maximum concentration (2×10^9 cells per ml), obtainable in cultures supplemented with hemin and NAD, after 18 h of incubation.

In the absence of NAD and in the presence of hemin, the cells grew at the same rate as in unsupplemented cultures, never producing visible evidence of growth after 24 h of incubation. The addition of NAD without hemin to simulated blood cultures, on the other hand, allowed the bacteria to grow at a higher rate than in unsupplemented cultures and, in a number of instances, to produce faint turbidity after 24 h of incubation. Because of this variability, the effect of adding NAD alone is not shown in Fig. 1.

We examined 100 negative blood cultures processed in the microbiology laboratory of the Methodist Hospital for their ability to support optimal growth rate of *H. influenzae* in the presence of hemin and NAD. All blood cultures consisted of two bottles; they had been incubated for 7 days with no visual evidence of growth and negative subcultures before being inoculated with *H. influenzae* at a concentration of 5 to 10 cells per ml. To one bottle, hemin and NAD were added, and both bottles were incubated overnight at 36°C and visually inspected for growth. All blood cultures with added hemin and NAD grew *H. influenzae* and produced visible turbidity. Blood cultures without added growth factors invariably remained crystal clear with no macroscopic evidence of growth; *H. influenzae* could be recovered from all bottles by subcultures.

DISCUSSION

In this communication, we report that the MGT of *H. influenzae* growing in simulated blood cultures supplemented with hemin and NAD is approximately one-third that of cells growing under identical conditions, but without added hemin and NAD.

In terms of bacterial numbers, such shortening of the generation time means that by the time one *H. influenzae* cell growing in conventional blood culture reaches its 10th doubling and 2^{10} (1,024) cells, the same cell growing in the presence of hemin and NAD will have undergone 30 doublings and have produced 2^{30} (1.1×10^9) cells.

Blood culture media used for the detection of *Haemophilus* bacteremia do not contain hemin or NAD, the assumption being that the patient's blood can serve as the sole source of the growth factors required by these bacteria.

The availability of the growth factors for haemophilic bacteria, however, depends on the lysis of erythrocytes and the concomitant release of their intracellular contents of NAD and hemoglobin into the medium.

In other experiments, not detailed in this study, erythrocytes freshly inoculated into a culture medium did not lyse during the first few hours of incubation, and the amount of hemoglobin released into the medium after 24 h of incubation indicated lysis of less than 0.5% of the total erythrocytes present in the medium.

It appears, therefore, that the lag phase in the multiplication of *H. influenzae* in simulated blood cultures without added growth factors was most probably due to the lack of accessible NAD and hemoglobin; conversely, the immediate increase in numbers of haemophilic bacteria in artificially lysed blood cultures (18) was most probably due to the sudden release of the growth factors from lysed erythrocytes into the medium.

H. influenzae grows well in the presence of

0.2 to 1.0 μg of NAD and 2 to 10 μg of hemin per ml of medium (9). According to Brinkley and Huber (5), the most suitable formulation for the growth of *Haemophilus* is Mueller-Hinton broth supplemented with 10 μg of NAD and hemin per ml. Our experiments showed (unpublished data) that *H. influenzae* can grow in the presence of 0.05 μg of NAD and 3.0 μg of hemin per ml, although at a greatly reduced rate.

The NAD plus NADP content of human erythrocytes is approximately 40 nmol/ml of whole blood (11), i.e., about 2.5 μg/ml of culture medium containing 10% (vol/vol) lysed blood. The heme content in the above medium would amount to approximately 500 μg/ml (based on the value of 12 to 15 g of hemoglobin per 100 ml of whole blood).

To achieve a concentration of 0.05 to 0.2 μg of NAD and 3 to 10 μg of heme per ml of blood culture, 2 to 8% and 0.6 to 2% of the erythrocytes ought to lyse, respectively.

With fewer than 0.5% erythrocytes lysing in 24 h, the concentration of growth factors released into the medium over this period of time should not exceed 0.012 μg of NAD and 2.5 μg of heme per ml. This concentration of NAD is far below that required for the optimal growth of *H. influenzae*. The actual concentration of NAD in the medium at any given time may be even lower than that calculated from the data on erythrocyte lysis, this being due to the presence in erythrocyte stroma of NAD glycohydrolase (NADase), which was reported to cause a decline in the NAD plus NADP content of human erythrocytes to about 20% of the original level after 7 days of incubation (14). The concentration of heme, on the other hand, may approach, after 5 to 10 h of incubation, the lower limit of the *H. influenzae* requirement for this growth factor. Thus, in some blood cultures, *H. influenzae* may appear to have no hemin requirement and grow overnight to produce faint turbidity when only NAD had been added to the medium.

In conclusion, NAD and, in many instances, also heme are the limiting factors which determine the growth rate of haemophilic bacteria in (simulated) blood cultures.

The conclusions drawn from this investigation are based on conditions chosen to simulate clinical situations. A concerted effort should be made to evaluate the usefulness of blood culture media supplemented with NAD and hemin for the rapid detection of *H. influenzae* bacteremia.

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