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False No-Growth Blood Cultures in Pneumococcal Pneumonia

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The growth of *Streptococcus pneumoniae* in commercial media containing ^{14}C-labeled substrates was studied experimentally; the results of blood cultures that were positive for *S. pneumoniae* over a 14-month period were analyzed to explain no-growth but radiometrically positive blood cultures from four patients with clinically diagnosed pneumococcal pneumonia. The growth of *S. pneumoniae* in aerobic blood culture vials resulted in a chocolate color in the medium. *S. pneumoniae* grew rapidly in both aerobic and anaerobic media, but $^{14}CO_2$ evolved from the metabolism of the labeled substrates was detected only in the aerobic culture vials. Radiometric detection lagged behind growth of the organisms and was accompanied by visual changes in the media. By 24 h, the viability of the culture was on the decline; radiometric readings remained positive even when the culture had died.

The isolation of bacteria from the blood has major clinical import. The earlier this happens, the better, so that appropriate therapy can be instituted. The initial detection of bacteremia can be accomplished by macroscopic, microscopic, and radiometric examination of blood cultures. We failed to isolate bacteria from blood cultures obtained from some patients with clinically documented pneumonia despite changes in the medium that suggested their presence. This led us to review our experience in the detection and isolation of *Streptococcus pneumoniae* from blood cultures and to examine the growth of *S. pneumoniae* in a radiometric blood culture system.

MATERIALS AND METHODS

Blood cultures from adult patients were processed by the BACTEC 460 system, and samples from pediatric patients were processed with Vacutainer supplemented peptone broth culture tubes (Becton Dickinson). The BACTEC 460 (Johnston Laboratories, Cockeysville, Md.) is a semiautomated system for the radiometric detection of bacteria in blood and other sterile fluid specimens. It measures $^{14}CO_2$ evolved from the bacterial metabolism of ^{14}C-labeled substrates present in the BACTEC culture medium. The amount of $^{14}CO_2$ liberated from the medium is expressed in growth index (GI) units. A GI reading of ≥ 30 in the aerobic vial and ≥ 20 in the anaerobic vial is considered positive and highly suggestive of bacterial growth.

Two media were used: the BACTEC culture vial type 6B, containing 30 ml of enriched peptone broth under a CO_2 atmosphere for aerobic cultures, and the 7B vial, containing 30 ml of prereduced enriched tryptic soy broth with additional supplements for anaerobic cultures, under an N_2-CO_2 atmosphere. Both contained sodium polyanethol sulfonate and ^{14}C-labeled substrates.

Using aseptic techniques, blood was drawn from each patient by an infusion set (Minicath 21; Deseret Pharmaceutical Co. Ltd., Sandy, Utah), and 3 ml was directly inoculated into each aerobic and anaerobic vial. The cultures were then taken to the microbiology laboratory and processed according to the manufacturer's suggested protocol. Cultures were examined at 4 h after collection at the earliest, and at 18 h at the latest. The aerobic vials were incubated on a shaker for the first 24 h, visually inspected, and tested on the BACTEC 460 twice a day for day 1 and then once daily for 7 days. The anaerobic vials were examined on the day after collection and daily thereafter. Many cultures were obtained late in the afternoon or at night. When cultures were radiometrically positive or showed visual changes, Gram-stained smears were prepared, and subcultures were made on Columbia base blood agar plates incubated aerobically and anaerobically (Gas Kit, Oxoid), chocolate agar plates incubated in CO_2 (GasPak, BBL Microbiology Systems) and cooked-meat medium. During the period of shaking it was not possible to assess turbidity of the aerobic vials, and only hemolysis could be assessed. All negative cultures were terminally subcultured on day 7. All cultures were incubated at 35°C.

Three recently isolated strains of *S. pneumoniae* were tested for growth in 30 ml of BACTEC aerobic 6B medium. Another strain, isolated from a patient's blood culture, was tested in a simulated blood culture system consisting of BACTEC aerobic 6B and anaerobic 7B vials to which 3.5 ml of citrated human blood was added. Control vials with blood were processed simultaneously. The vials were inoculated with 1 ml of varying dilutions of 3-h Mueller-Hinton broth cultures of the test strains, grown at 35°C in CO_2. The inoculum size was estimated by plating out 0.1 ml of 10-fold dilutions of the Mueller-Hinton broth dilution used to inoculate the vials. The culture vials were processed on the BACTEC 460 as described, except

that each vial was tested at 2, 4, 6, 12, 24, 48, and 72 h. Counts of colony-forming units were obtained by spreading 0.1-ml samples of each culture over the surface of Columbia base blood agar plates, which were incubated for 48 h at 35°C in CO_2.

In addition, 0.3 ml of each dilution inoculated in the BACTEC vials was inoculated into tubes containing 10 ml of brain heart infusion broth and examined at 6 or 12 h and at 24, 48, and 72 h.

RESULTS

During a 14-month period between January 1979 and February 1980, *S. pneumoniae* was isolated from 18 patients (7 pediatric and 11 adults), representing 7% of the significant bacteremias due to single organisms in our hospital. Blood cultures from adult patients were processed with the BACTEC system. In these patients, the interval between collection of blood and the detection of positive GI readings ranged from 6 h to 4 days, with a median of 17 h (9 of 11 by 22 h). The medium in some of the GI positive aerobic vials had become the color of chocolate agar. In others, hemolysis was observed at first, and on continued incubation the medium became a chocolate color. In the visually positive anaerobic vials, hemolysis was evident and the color of the medium was a dark red. The GI never exceeded 20. The Gram-stained smears showed organisms ranging from typical gram-positive diplococci to gram-variable and gram-negative forms. In one patient, *S. pneumoniae* was isolated only from the anaerobic vials, which showed hemolysis at 4 days, with a GI of 5.

Blood cultures from four patients with clinically and radiologically diagnosed pneumococcal pneumonia were negative, despite positive GI readings (Table 1). None of them was receiving antibiotics or immunosuppressants at the time the cultures were taken. The interval between collection and initial testing ranged from 14 to 18 h, at which time the positive aerobic vials had GI readings of >100 and the culture medium had changed overnight to a muddy chocolate color. Some of the cultures from one of these four patients had GI readings of 32 to 48. The medium in these vials did not change color. This patient had a leukocyte count of 145 x 10^9 per liter. A positive Quellung test confirmed pneumococcal bacteremia in another.

Ninety-one percent of blood cultures submitted during this period were negative. After several days, some of the negative cultures became hemolyzed and were a dark plum color. This color was easily distinguished from the chocolate color seen with positive *S. pneumoniae* cultures.

In the experiments, all inocula grew rapidly, producing confluent growth on subculture of 0.1 ml from the vials within 24 h. Representative experiments are shown in Table 2. There was a definite lag before bacterial growth was detected radiometrically in the aerobic vials without blood. The growth indices remained low for up to 12 to 24 h. Two of the strains, one of them cultured from the empyema of patient no. 2, were no longer viable at 24 h, although the GI was positive for up to 72 h.

In the simulated blood culture inoculated with a clinical isolate from blood, a lag between growth and BACTEC detection was again observed in the aerobic vials. The GI was positive at 12 h, at which time the medium was a chocolate color. In the anaerobic vials, the GI remained negative and hemolysis was present at 24 h. The bacteria in the aerobic BACTEC vials started to die out after 24 h, whereas subcultures from the anaerobic vials yielded heavy or confluent growth of *S. pneumoniae* up to 72 h.

DISCUSSION

Pneumococci are known to be subject to self-destruct processes in liquid cultures which first render the cells gram negative and later bring about lysis of the culture. The activity of autol-

TABLE 1. *Relevant findings present with no-growth blood cultures*

Case no.	Leukocyte count (×10^9 per liter)	BACTEC 6B culture vial results		Other source of *S. pneumoniae*
		GI Units[a]	Gram-stained smear	
1	13.3	203	Gram-positive diplococci Quellung test positive	Sputum
		18, 19	NOS[b]	
2	3.3	211, 226, 229	NOS	Sputum, pus from empyema
3	19.8	219	Gram-negative cocco-bacilli	
4	145	128	Gram-negative diplococci	Sputum, pharynx
		23, 32, 45, 45, 48	NOS	

[a] Highest GI from individual vials.
[b] NOS, No organisms seen.

TABLE 2. *Growth of S. pneumoniae in the BACTEC radiometric system*

Culture	Inoculum	GI (CFU/ml)[a] at time (h):						
		2	4	6	12	24	48	72
Strain								
A	4.0×10^2, aerobic	15 (160)	13 (300)	10 (+)	71 (++)	98 (0)	79 (0)	62 (0)
B	5.0×10^2, aerobic	15 (10)	9 (20)	11 (80)	9 (2,090)	40 (+)	88 (+)	155 (+)
C	5.7×10^3, aerobic	14 (230)	12 (2,040)	12 (++)	81 (++)	89 (0)	69 (0)	54 (0)
Simulated blood culture	4.3×10^3, aerobic	6 (850)	8 (+)	7 (++)	103* (++)	208* (+)	88* (8,100)	45* (40)
	Anaerobic	1 (900)	3 (+)	3 (++)	12** (++)	11*** (++)	8*** (++)	1*** (+)
Controls without blood	Aerobic	7 (530)	7 (3,950)	7 (+)	20 (++)	50 (++)	125 (250)	
	Anaerobic	3 (400)	4 (1,470)	1 (2,080)	3 (870)	4 (++)	16 (++)	

[a] CFU, Colony-forming units. Growth: +, heavy, nonconfluent; ++, confluent. Appearance: *, chocolate; **, slight turbidity; ***, hemolysis.

ysins such as *N*-acetyl-muramyl-L-alanine amidase (8), the production of hydrogen peroxide by superoxide dismutase (9), the lack of catalase, and the production of a low pH are factors affecting the viability of the culture.

Pneumococci produce an intracellular hemolysin which is liberated by autolysis. This pneumolysin is active against human, sheep, rabbit, and horse erythrocytes. On blood agar, alpha-hemolysis is produced under aerobic conditions, and some strains produce beta-hemolysis under anaerobic conditions (4). Hydrogen peroxide converts free hemoglobin to brown methemoglobin. Spectrophotometric analysis of the medium from an aerobic vial showed a peak at 630 nm corresponding to that of methemoglobin (S. Wong, personal communication). This was absent in the anaerobic vials. This explains the chocolate color observed in the culture-positive aerobic vials. It is of interest that the blood-streaked sputum of patients with pneumococcal pneumonia is classically described as rusty.

Our observations of blood cultures positive on subculture showed that the change of the medium to a chocolate color accompanied by a positive radiometric detection in aerobic culture vials indicated the growth of *S. pneumoniae*. The experimental findings substantiate this. We did not observe this color change in cultures positive for other organisms. Despite such evidence of bacterial growth, paradoxically, *S. pneumoniae* was not recovered from these four patients. False-positive radiometric readings have been described in association with high leukocyte counts (1, 3, 5). Four of six aerobic 6B cultures obtained from one of our patients, who had a high leukocyte count, had positive GI readings. Only one, with a GI of 128, showed evidence of the growth and death of bacteria. By contrast, the other 6B vials showed no change visually, had GI readings of 32 to 48, and had no other findings to suggest bacterial growth. In this and other reported cases, false-positive GI readings associated with a high leukocyte count have not exceeded 60. We believe that the chocolate-brown color change of the medium, combined with radiometric change in aerobic 6B blood cultures obtained from patients clinically diagnosed as having pneumococcal infection, is presumptive evidence that *S. pneumoniae* has grown and subsequently died.

Since only 6 to 20% of blood cultures taken are positive (2, 10), the radiometric detection of bacterial growth sorts out the positive cultures, resulting in the elimination of the tedious and labor-intensive aspects of examining the negative cultures by Gram stain and subculture. The time interval from inoculation to radiometric detection is proportional to the size of the initial inoculum. This period is shorter for gram-negative organisms than for streptococci; with inocula as low as 10 organisms this may take up to 15 h (7), by which time most organisms will grow to 10^7 colony-forming units per ml. Our experiments clearly show a lag between growth of inocula in aerobic vials and their radiometric detection, corroborating the findings of Washington and Yu (6).

The radiometric examination of anaerobic vials was not helpful in the early detection of pneumococcal bacteremia. They were not always culture positive when the aerobic vials were positive. Though the pneumococcus grew very well, the GI was well below threshold, and

if only the anaerobic vial was positive, detection was limited to visual inspection, leading to a delay in the detection of bacteremia.

It is possible that more frequent testing of the aerobic vials might detect a rise in GI readings or visual change to indicate bacterial growth sooner. The equipment turnaround time does not permit more than two readings on each vial on the BACTEC during normal working hours when dealing with daily workloads of 250 or more vials. More frequent examination would reduce the cost effectiveness of the machine. Even then, because of the lag between growth and radiometric detection, it may not solve the problem of the failure of the BACTEC to detect *S. pneumoniae* in some cultures before the organisms die. This is also a problem of the conventional system of blood culturing. Since the blood culture may be the only specimen from which pneumococci are recovered, it would be prudent to examine blood cultures from patients suspected of pneumococcal infection by visual inspection, Gram stain, and subculture no later than 12 h after collection.

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