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Rapid Detection of Bacterial Growth in Blood Cultures by Bioluminescent Assay of Bacterial ATP

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A method for rapid detection of bacterial growth in blood cultures by bioluminescent assay of bacterial ATP was developed. Samples from blood cultures were treated with a blood-lysing detergent combined with an ATP-hydrolyzing enzyme to destroy blood cell ATP. Blood cell ATP which was bound to cell debris and escaped the ATPase activity was then separated from the bacteria on Percoll density gradients. Bacterial ATP from the bacterial layer was determined by the firefly bioluminescence system. In simulated blood cultures inoculated with 10 CFU of bacteria per ml of blood, bacterial ATP levels exceeded the detection limit (10^{-10} M) after 6 to 10 h of incubation. This ATP level corresponds to approximately 10^4 CFU of bacteria per ml.

The bioluminescent assay of bacterial ATP has been used for rapid detection of bacteriuria (1–6). In this assay, conditions are arranged to result in a light emission proportional to the ATP concentration.

A major problem in the assay of bacterial ATP in clinical specimens is the occurrence of large amounts of nonbacterial ATP from somatic cells present in the specimens (2). This study deals with possible ways to reduce blood cell ATP to develop a method for rapid detection of bacterial growth in blood cultures.

MATERIALS AND METHODS

Analytical equipment. Light emission from the bioluminescent assay was measured in a 1250 Luminometer (LKB-Wallac, Turku, Finland) and registered on a 1250 Display (LKB-Wallac). The extraction of bacterial ATP was performed in a LKB-Biocal 2073 incubator (LKB Products, Bromma, Sweden).

The gradient centrifugation was performed in a Sorvall Superspeed RC2-13 centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) with polycarbonate tubes and a SE-12 rotor (also from Sorvall).

Analytical reagents. An ATP monitoring kit (LKB-Wallac) was used in the assay of ATP Apyrase (purified grade I) was purchased from Sigma Chemical Co., St. Louis, Mo. Isopycnic separation was performed on Percoll gradients (Pharmacia Fine Chemicals, Uppsala, Sweden). All other reagents were of analytical grade.

Blood culture. A two-phase blood culture system was used. The culture bottles contained 20 ml of Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) and brain heart infusion agar (Difco Laboratories, Detroit, Mich.). The Trypticase soy broth was supplemented with 100 U of penicillinase (Difco) per ml, 1% IsoVitaleX (BBL), and 0.08% $NaHCO_3$. Brain heart infusion agar (Difco) was supplemented with 0.05% KNO_3, 0.02% glucose, 0.004% hemin, and 0.05% Liquoid (Hoffmann-La Roche Inc., Nutley, N.J.). The blood cultures were inoculated with 2 ml of blood.

Extraction of blood cell ATP. A 50-μl sample from a blood culture bottle, containing 10% (vol/vol) fresh blood, was pipetted into 500 μl of boiling 0.1 M Tris buffer (pH 7.75) containing 2 mM EDTA. After heating for 90 s, the extracts were cooled to room temperature before assay of blood cell ATP.

Reduction of blood cell ATP with fractionated centrifugation and treatment with Triton-apyrase. To separate blood cells from bacteria, 1 ml from an inoculated blood culture was centrifuged at 250 × *g* for 5 min. After the centrifugation, a 50-μl sample from the supernatant was incubated for 10 min at 37°C with 50 μl of a Triton-apyrase solution consisting of 0.04% apyrase and 0.2% Triton X-100 made up in supplemented Trypticase soy broth.

Elimination of blood cell ATP with gradient centrifugation and treatment with Triton-apyrase. To reduce blood cell ATP, 1 ml from an inoculated blood culture bottle was incubated for 10 min at 37°C with 1 ml of the Triton-apyrase solution described above. Separation of blood cell debris from bacteria was performed on a Percoll gradient. A standard isoosmotic Percoll stock solution was prepared by diluting 90 ml of undiluted Percoll with 10 ml of 1.5 M NaCl. A 35% (vol/vol) solution was made by combining 35 ml from the standard isoosmotic Percoll solution with 65 ml of 0.15 M NaCl.

A continuous gradient was generated by centrifugation of 4 ml of 35% (vol/vol) Percoll solution for 20 min at 30,000 × *g*. Density separation was achieved by layering the Triton-apyrase–treated blood sample (2 ml) on top of the gradient and centrifuging at 400 × *g* for 20 min. The position of the bacterial layer can be determined by density marker beads (Pharmacia Fine Chemicals AB) or by gradient centrifugation of a bacterial culture with a bacterial count exceeding 10^6 CFU/ml, which gives a visible layer at a specific region of the gradient.

To eliminate remaining blood cell ATP, 50 μl from

the bacterial layer was incubated with 50 μl of apyrase solution for 10 min at 37°C.

Extraction of intracellular bacterial ATP. After the elimination of blood cell ATP, bacterial ATP was extracted with the same procedure as that used for extraction of blood cell ATP. The extraction inhibits the action of the apyrase and disrupts the bacteria, thus releasing their ATP content.

Luciferase assay of ATP. Luciferase reagent (100 μl) was added to each extract (500 μl), and the light intensity was recorded.

Calculation of assay results. Sample ATP levels were calculated by using assays of standard amounts of ATP as a reference, and correction for background luminescence was made. An internal standard technique was used to correct for inhibition of the luciferase reaction in the extracts, i.e., the addition of known amounts of ATP to the extracts.

Determination of bacterial viability. Bacterial numbers were determined as CFU per milliliter by plating after serial dilution.

Simulated blood cultures. Blood drawn from healthy individuals was inoculated with bacteria (10 CFU/ml), and 2 ml was added to blood culture bottles. One strain each of *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus agalactiae*, and *Streptococcus faecalis* was used. All strains were clinical isolates. The blood culture bottles were incubated at 37°C, and samples for viable count, analysis of bacterial ATP, Gram staining, and subculture (10 μl) on hematin agar plates were taken every hour for 12 h.

The entire bioluminescent assay of bacterial ATP in blood cultures is outlined schematically in Fig. 1.

RESULTS

The blood cell ATP level in blood culture bottles containing 10% (vol/vol) fresh blood was 10^{-4} M after extraction with boiling Tris buffer. If samples from blood cultures were lysed with detergent (Triton X-100) and treated with the ATP hydrolyzing enzyme apyrase, this ATP level was reduced to 10^{-6} M. Fractionated centrifugation of samples from the blood cultures at 250 × *g* reduced the blood cells in the supernatant. The remaining ATP level in the supernatant after extraction was 10^{-6} M. If the supernatant was treated with detergent and ATPase, no ATP could be detected before extraction. However, after extraction with boiling Tris buffer, blood cell ATP (10^{-8} M) reappeared. This background of blood cell ATP (10^{-8} M) was too high to be accepted, because the amount of bacteria in simulated blood cultures had to exceed approximately 10^6 CFU/ml before bacterial growth was detectable. Furthermore, the yield of bacteria in the supernatant fluid after centrifugation varied with different bacterial strains (Table 1).

To overcome the background of blood cell ATP (10^{-8} M), a more complete separation of blood cells and bacteria was necessary. To achieve this, samples from the blood culture bottles were subjected to density gradient centrifugation. However, the bacterial layer and the blood cell layers were too close in the gradient after centrifugation, due to the small difference

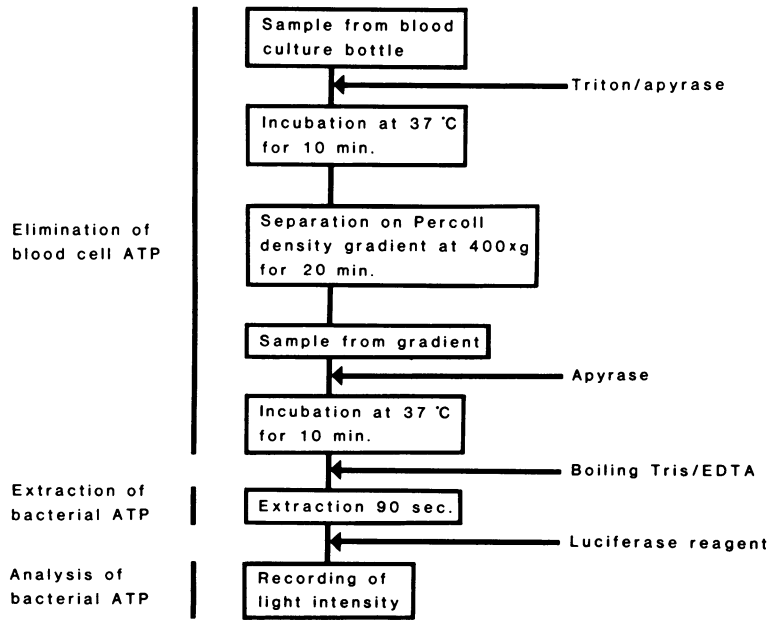

FIG. 1. Schematic outline of the bioluminescent assay of bacterial ATP in blood cultures.

TABLE 1. Comparison of yields of bacteria in blood cultures incubated overnight and molarities of ATP in blood cultures, in supernatants after fractionated centrifugation at 250 × *g*, and in gradients after Percoll density separation

Bacterial strain	Yield of bacteria in:				
	Blood culture	Supernatant		Percoll gradient	
	CFU/ml × 10^9	CFU/ml × 10^9	M ATP × 10^{-6}	CFU/ml × 10^9	M ATP × 10^{-6}
Escherichia coli	2.9	2.1	6.0	4.4	22
Klebsiella pneumoniae	3.6	1.3	12.3	5.2	12
Proteus mirabilis	5.2	3.8	4.2	12.3	28
Pseudomonas aeruginosa	3.7	1.7	4.3	6.2	9.1
Staphylococcus aureus	0.43	0.14	0.19	1.4	43
Staphylococcus epidermidis	0.53	0.01	0.06	2.1	37
Streptococcus agalactiae	1.0	0.13	1.3	4.4	54
Streptococcus faecalis	2.5	0.76	2.2	4.5	35

in their densities. This was circumvented by lysing blood with detergent before gradient centrifugation. After this treatment, a satisfactory separation between bacteria and cell debris was achieved. When samples from blood cultures without bacteria were treated with detergent and ATPase and were subjected to gradient centrifugation, no blood cell ATP could be detected in samples from the specific region of the gradient in which the bacterial layer was known to be found. The detection limit of ATP was 10^{-10} M, due to the background luminescence from the luciferase reagent and the sensitivity of the equipment. The bacterial yield in samples from the bacterial layer in the gradient was equal to or greater than that in the blood culture bottles, due to the zone-sharpening effect of the centrifugation (Table 1).

In simulated blood cultures, ATP levels exceeded the detection limit (10^{-10} M) after 6 to 10 h of incubation (Fig. 2 and 3; Table 2). This ATP level corresponded to approximately 10^4 CFU of bacteria per ml (Fig. 2 and 3). The time of detection with Gram staining was 1 to 2 h later (Table 2). Subculture detected the bacteria after 22 to 25 h of incubation, including 18 h of incubation of agar plates (Table 2). The approximate detection limits of bacteria with subculture

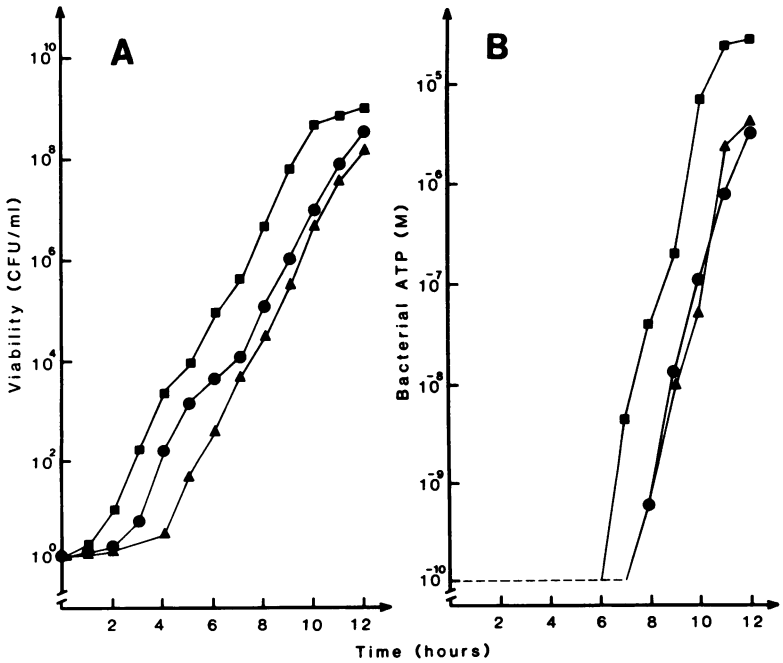

FIG. 2. Growth of *E. coli* (■), *P. mirabilis* (●), and *S. aureus* (▲) in simulated blood cultures monitored by viability (CFU per milliliter) (A) and bacterial ATP (molarity) (B). The cultures contained 10% (vol/vol) blood.

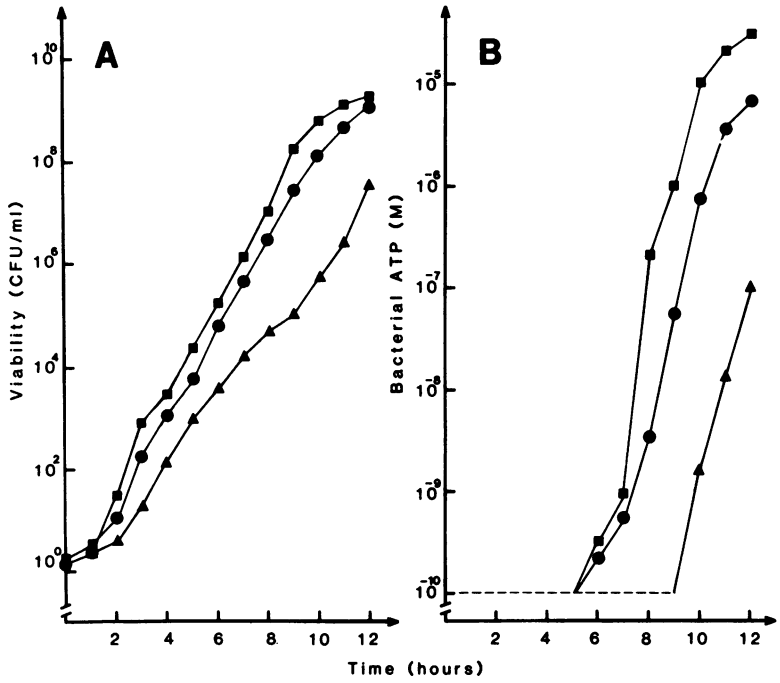

FIG. 3. Growth of *K. pneumoniae* (■), *S. faecalis* (●, and *P. aeruginosa* (▲) in simulated blood cultures monitored by viability (CFU per milliliter) (A) and bacterial ATP (molarity) (B). The cultures contained 10% (vol/vol) blood.

and Gram staining were 1×10^3 and 5×10^5 CFU/ml, respectively.

DISCUSSION

ATP levels (10^{-10} M) corresponding to 10^4 CFU of bacteria per ml can be detected with reagents and equipment used in the present bioluminescent assay. However, a major problem in the assay of bacterial ATP in blood cultures is the presence of large amounts of blood cell ATP (10^{-4} M). A selective method for distinguishing bacterial and nonbacterial ATP in urine specimens has been presented (5). The method involved incubation of samples with detergent and ATPase. This treatment destroyed ATP in suspensions of various human cells but did not affect the ATP content in bacteria (5). When applied to blood cultures, the detergent-ATPase treatment did not sufficiently eliminate the blood cell ATP. The remaining level of blood cell ATP (10^{-6} M) makes it impossible to detect less than 10^8 CFU of bacteria per ml. Centrifugation ($250 \times g$) of samples to settle most of the blood cells into a pellet, followed by treatment of the supernatant with detergent and ATPase, gave a further decrease of the background of blood cell ATP. However, the detergent did not

TABLE 2. Detection time of bacterial growth in blood culture bottles inoculated with 10 CFU of bacteria per ml of blood with subculture, bioluminescent assay, and Gram staining

Bacterial strains	Detection time (h) with:		
	Subculture[a]	Bioluminescent assay	Gram staining
Proteus mirabilis	23	8	9
Escherichia coli	22	7	8
Klebsiella pneumoniae	23	6	8
Pseudomonas aeruginosa	23	10	12
Streptococcus faecalis	22	6	8
Staphylococcus aureus	25	8	9

[a] Detection time with subculture includes incubation time of blood cultures plus 18 h of incubation of agar plates.

release all blood cell ATP, since a background ATP level (10^{-8} M) reappeared after the extraction procedure with boiling Tris buffer. ATP probably remains bound to blood cell membranes and escapes the ATPase activity. Furthermore, bacteria were supposed to remain in the supernatant after centrifugation at 250 × *g*, but the bacterial yield in the supernatant varied with different bacterial strains, probably due to their tendency to form aggregates (Table 1).

A better separation of blood cells and bacteria was necessary to overcome the background of blood cell ATP (10^{-8} M). Density gradient centrifugation with Percoll has been used successfully in the separation of mammalian and bacterial cells (Percoll methodology and applications, Pharmacia Fine Chemicals). To achieve the best separation of blood cells and bacteria, it is convenient to use a concentration of Percoll which has a density similar to that of the bacteria. A continuous gradient made by a 35% Percoll solution fulfilled this requirement. The small difference between the densities of blood cells and bacteria necessitated lysing blood cells with detergent before gradient centrifugation. The released ATP was eliminated by ATPase. The ATP which was bound to cell debris and escaped the ATPase activity was separated from the bacteria during the gradient centrifugation. By this procedure, we can use the full sensitivity (10^{-10} M) of the bioluminescent assay with the reagents and equipment used in this study. The yields of bacteria after gradient centrifugation were equal to or greater than those in direct samples from blood culture bottles, i.e., the bacteria were concentrated in the bacterial layer in the gradient.

The detection time of bacterial ATP in simulated blood cultures inoculated with 10 CFU of bacteria per ml of blood varied between 6 and 10 h. At the time of detection, the amount of bacteria just exceeded 10^4 CFU/ml, corresponding to the detection limit of bacterial ATP (10^{-10} M). No bacterial layer was visible in the gradient until the bacteria exceeded approximately 10^6 CFU/ml. Thus, bacterial growth is detectable with the bioluminescent assay in a relevant sample from the gradient, even though no bacterial layer is visible.

Direct microscopic examination of blood cultures by Gram staining required approximately 5 × 10^5 CFU of bacteria per ml before detection was possible. This was obtained 1 to 2 h later than the detection of bacteria with the bioluminescent assay. With 10-μl samples from the blood culture bottles, subcultures on hematin agar plates detected bacterial growth when the number of bacteria exceeded 10^3 CFU/ml. However, the subcultures had to be incubated overnight before there was detectable growth, and then the blood culture bottles showed visible growth.

We conclude that the bioluminescent assay of bacterial ATP is a rapid, objective, and sensitive method for early detection of bacterial growth in blood cultures.

ACKNOWLEDGMENTS

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