Growth and Endotoxin Production of Yersinia enterocolitica and Enterobacter agglomerans in Packed Erythrocytes

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Since 1987, the Centers for Disease Control investigated six cases of transfusion-associated sepsis. All six patients developed septic shock after receiving units of packed erythrocytes (PRBCs) contaminated with Yersinia enterocolitica (five patients) and Enterobacter agglomerans (one patient); three of the blood recipients died. We studied the growth and endotoxin production of Y. enterocolitica and E. agglomerans in units of PRBCs stored at 4°C for 60 days. When PRBCs were inoculated with 0.1 to 1.0 CFU of these organisms per ml, both Y. enterocolitica and E. agglomerans entered log-phase growth 2 to 3 weeks after inoculation; generation times were 15 and 22 h, respectively. Endotoxin was first detected at 3 weeks following inoculation, and the concentration paralleled the log phase of growth of the strains tested. These data show that prolonged storage of PRBCs at 4°C provides conditions that allow these two organisms to grow and subsequently produce high concentrations of endotoxin.

The occurrence of septicemia as a result of receiving blood products contaminated with bacteria was common during the early years of transfusion therapy (8, 11, 13, 14). The major risk factor for blood product contamination was considered to be inadequate aseptic technique during collection of the blood. After sealed, disposable collection systems were developed and storage of blood at 4°C became standard, sepsis caused by contaminated blood products became a rare event. Since 1979, there have been 17 reported cases (11 fatalities) of gram-negative bacteremia associated with the use of whole blood or packed erythrocytes (PRBCs) (2, 5, 6, 9, 12, 19, 21, 23, 24, 26, 27); Pseudomonas fluorescens, P. putida, Yersinia enterocolitica, and Campylobacter jejuni have been implicated.

We recently investigated four episodes of Y. enterocolitica sepsis (4) and one case of Enterobacter agglomerans sepsis associated with the transfusion of PRBCs. We are currently investigating a fifth case of transfusion-associated Y. enterocolitica sepsis. All of the recipients developed septic shock after receiving the contaminated blood. Three of the patients who received Y. enterocolitica-contaminated blood died as a result of receiving the contaminated PRBCs. The results of the investigation showed that in at least two cases of Y. enterocolitica infection, bacterial contamination probably resulted from low-level asymptomatic bacteremia in the blood donors. In all six cases, the units of PRBCs were stored at 4°C for periods of 21 days.

The purpose of these laboratory studies was to determine the growth rates and amounts of endotoxin production of Y. enterocolitica and E. agglomerans in similar units of PRBCs stored at 4°C.

MATERIALS AND METHODS

Three units of freshly donated human PRBCs stored in adenine saline (preservation solution and citrate phosphate dextrose anticoagulant solution; CPDA-1) were obtained from the Centers for Disease Control serum bank. A 2-ml sample was aseptically drawn from each of the units through a sampling site coupler (Fenwal Laboratories, Deerfield, Ill.) for verification of sterility and absence of endotoxin. Two units were inoculated with Y. enterocolitica, and the third unit was inoculated with E. agglomerans to achieve a final concentration of approximately 1 CFU/ml. Both test organisms had been isolated from case patients, grown on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) slants, harvested, and suspended in sterile saline for inoculation. After inoculation, each unit of PRBCs was rocked for 5 min to mix the inoculum. A time zero (t₀) sample was obtained from each unit for bacterial and endotoxin assays. The three units of PRBCs were stored at 4°C and sampled for bacteria and endotoxin over a period of 60 days.

Samples were aseptically collected through the Fenwal sampling site coupler with sterile 10-ml syringes fitted with a 23-gauge 1.5-in. (1 in. = 2.54 cm) needle (Becton Dickinson and Co., Rutherford, N.J.) during the first 3 weeks of the study. 6-ml samples were collected for analysis three times a week: 5 ml for bacterial culture and 1 ml for endotoxin analysis. Bacteriologic assays were performed by spreading 1-ml samples onto each of five Trypticase soy agar II plates with 5% sheep blood (BBL Microbiology Systems) and incubated at 30°C for 24 to 48 h. During the remaining 6 weeks of the study, 2-ml samples were collected: 1 ml for endotoxin analysis and 1 ml for bacterial culture. The 1-ml PRBC sample for bacterial culture was serially diluted by a factor of 10 (10⁻¹, 10⁻², . . . , 10⁻⁶) in sterile saline and assayed by membrane filtration with 47-mm-diameter cellulose nitrate 0.45-μm-pore-size white grid filters (Micro Filter Systems, Dublin, Calif.). Filters were aseptically placed onto Trypticase soy agar II plates and incubated at 30°C for 24 to 48 h. Colonies were counted, and a growth curve for each unit of PRBCs was constructed. Generation time at 4°C (i.e., the time required for the bacterial population to double) was calculated for each organism.

For endotoxin analysis, PRBC samples (from each of the three units) were placed in endotoxin-free containers and microcentrifuged for 3 min. The plasma was then removed, diluted 1:10 with sterile 0.9% saline, and heated at 70°C for
10 min to remove endotoxin inhibitors (7, 10, 16, 25). The sample was again diluted 1:10 with saline to make the final sample dilution 1:100. Detection and quantitation of endotoxin were done by the Limulus amebocyte lysate turbidimetric assay (LAL-5000; Associates of Cape Cod, Woods Hole, Mass.) (15, 22; Associates of Cape Cod, LAL Update 52, 1987). A control standard endotoxin (lot 36; Associates of Cape Cod) was used for all standard curves, positive controls, and product inhibition determination (15, 22).

RESULTS

Bacterial concentrations in the three units of PRBCs were 0.1 to 1.0 CFU/ml at t₀. Concentrations of both Y. enterocolitica and E. agglomerans remained in the lag phase for 7 days in the PRBCs stored at 4°C and did not enter the log phase of growth until 2 to 3 weeks postinoculation (Fig. 1). During the log-phase period of growth, the generation times for Y. enterocolitica and E. agglomerans were 15 and 22 h, respectively. Bacterial growth in all units reached the stationary growth phase 5 to 6 weeks after inoculation with viable colony counts of 10⁶ CFU/ml. Colony counts remained stable for the remainder of the 9-week study.

Endotoxin was not detected in any of the units of PRBCs until they had been stored at 4°C for 21 days, at which time concentrations increased rapidly, the rise paralleling the log-phase growth curve (Fig. 2). Mean endotoxin levels reached a maximum concentration of 240 to 600 ng/ml (2.38 to 2.78 log₁₀ U) approximately 6 weeks after inoculation. After week 6 of storage, endotoxin levels began to decrease. Inhibition studies using a control standard endotoxin did not detect inherent inhibition in the plasma under study.

DISCUSSION

Results of previous studies on the growth of Y. enterocolitica in milk stored at 4°C were similar to those of our study: generation times were 16.8 to 22.9 h, and bacterial counts were 10⁶ CFU/ml within 21 days (1). Studies by Stenhouse and Milner (1982) also demonstrated that Y. enterocolitica could reach concentrations of 10⁶ CFU/ml at 21 days after inoculation of a fresh unit of whole blood stored at 4°C (24). However, the concentrations of inocula at t₀ were approximately 2 logs greater than in our study. When our results were compared with those of a similar study of P. fluorescens (9), the growth of Y. enterocolitica was much slower during the first 7 to 10 days but reached a stationary-phase titer about 2 logs higher after 28 days. In our study, E. agglomerans showed slower growth and a longer log phase of growth and generation time than did Y. enterocolitica but reached stationary-phase titers similar to those of P. fluorescens (9) and Enterobacter and Pseudomonas species (3).

Previous data have suggested that endotoxin doses that exceed 1.0 ng/kg of body weight (threshold pyrogenic dose) produce pyrogenic reactions in patients (20). The levels achieved here, 240 to 600 ng/ml, have been shown to be fatal in animal models (3). These levels would theoretically produce a severe pyrogenic reaction. Why the endotoxin concentrations decreased after week 6, even though the bacterial counts remained constant at 10⁶ CFU/ml, is not completely understood. The decrease could be the result of chemical binding of the endotoxin to plasma (17), binding to the PRBCs (18), or possibly binding to the blood bag. Because all three units of PRBCs displayed similar endotoxin concentration decreases and no inhibition was found in the plasma under study, such a decrease, as determined by the Limulus amebocyte lysate assay, appears real. This decrease in endotoxin concentrations should be considered when endotoxin assays are conducted on blood products more than 5 to 6 weeks old.

Our results confirm that low-level contamination (e.g., 0.1 CFU/ml), whether from a donor with asymptomatic bacteremia or some other source, can result in a blood product (even if stored at 4°C) containing high concentrations of endotoxin and bacteria after 2 to 3 weeks. The demonstrated abilities of Y. enterocolitica and E. agglomerans and other psychrophilic organisms to grow and produce high concentrations of endotoxin at 4°C make these organisms especially hazardous in blood and blood products subjected to prolonged storage at 4°C. However, the occurrence of such related transfusion reactions is a relatively rare event. This study demonstrates that storage at 4°C does not ensure the sterility of blood products, absence of microbial growth, or endotoxin production.

LITERATURE CITED