Inhibition of Bacterial Growth by Granulocytes Measured by an Automated Technique

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ABSTRACT

Granulocytes, collected by several methods, were assayed for antibacterial activity utilizing a technique originally developed for automated antibiotic susceptibility testing. The granulocytes were incubated with either *Escherichia coli* or *Staphylococcus* for one hour at 37°C and were then separated from the suspension by gentle centrifugation at 170g. The bacteria remaining in the supernatant broth were incubated in culture medium (eugonic broth) and their growth density was compared with diluted controls (without granulocytes) by measurement in an Autobac I (an automated nephelometer).

Measurements of the density of growth at 30 minute intervals showed a marked delay in the development of density suggesting only small numbers of organisms remained after incubation with granulocytes. Once density was measurable, the rate of growth appeared similar to controls. After 2.5 hours in the Autobac I, the density of growth was used to determine the inhibitory effect of granulocytes. The density of growth was inversely related to the concentration of granulocytes present during the preincubation phase. Filtered granulocytes showed significantly lower inhibitory effect than centrifuged granulocytes. This rapid, inexpensive method of determining microbial growth appears to be adaptable as a measure of granulocyte function.

Introduction

Antibacterial activity is one measure of function being utilized at present to evaluate granulocytes which are collected by several techniques of leukapheresis for transfusion. In addition, granulocytes stored under various conditions are being evaluated for functional survival, in part, by their ability to kill bacteria.\(^1, 2, 4, 5, 6, 9, 12, 14\) Techniques of quantitating bacterial growth by pour plate and counting colonies are both time consuming and imprecise.\(^6, 10, 13\)

The present study describes a simple, rapid and reproducible technique for determining bacterial growth after exposure to granulocytes. The technique is patterned after a recently developed method for rapid antibiotic susceptibility testing.\(^10\) It is applied to compare the bactericidal capacity of granulocytes collected
from normal donors by filtration\(^1\) or by intermittent flow centrifugation using hydroxyethyl starch as sedimenting agent.\(^7\)

**Methods and Materials**

Test microorganisms, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* 502A were obtained from standard cultures maintained in the Microbiology Division of the Department of Laboratory Medicine at the University of Connecticut. A loop (stylet) was used to transfer freshly plated organisms to a vial of sterile 0.85 percent sodium chloride. The concentration of organisms was adjusted to between 20 and 30 million organisms per ml using the nephelometer of the Autobac I.*

Two ml of saline suspended microorganisms were diluted in 15 ml of eugonic broth* making a final concentration of about 2.25 million organisms per ml. Diluted standards were prepared by further doubling dilutions in eugonic broth to provide a standard curve. These standards were incubated at 37°C for one hour and then centrifuged at 170 g for 10 minutes to match the procedure used when evaluating granulocytes. During development of this procedure supernatant broth was dispensed into the cuvets of the Autobac I and further incubated with agitation in the incubator of the Autobac I. At 30 minute intervals, the cuvets were removed from the incubator and placed in the carrier of the nephelometer. The cuvets were read automatically in sequence. The cuvet was positioned in the Autobac photometric device in which the light scattered at a 35° angle to the incident beam was received and measured by the photodetector. The photodetector produced a current output directly proportional to the intensity of the light reaching it. Over wide limits, the change in this value was directly proportional to the number of microorganisms per unit volume and was used as a sensitive measure of changes in microbial concentration. A standard curve was constructed relating the change in voltage at fixed time intervals to the number of organisms in the dilution of standard culture.

Control granulocytes (CG) were obtained from blood collected in heparin† without preservative. A 30 ml plastic syringe was rinsed with heparin and 10 to 15 ml of blood were obtained by clean venipuncture. The heparinized blood was mixed with 6 percent Dextran in equal volumes. After sedimentation for 30 minutes, the granulocyte rich plasma was expressed from the vertically held syringe, and the granulocytes were centrifuged at 170 g for 10 minutes and suspended in TC 199‡ to a final count between 2 and 10 million cells per ml.

Test granulocytes (TG) were obtained from normal donors by two techniques of leukapheresis, intermittent flow centrifugation (IFC) using hydroxyethyl starch (HES) to improve separation of granulocytes from red cells, and continuous flow filtration (CFF) through nylon filters using heparin as anticoagulant while the donor was undergoing leukapheresis, and citrate anticoagulant with 20 percent plasma in saline for elution of granulocytes from the filters as previously described.\(^9\) Samples (one to two ml) of TG were centrifuged and suspended in TC 199 in the same fashion as CG.

To determine the effect of granulocytes on bacterial growth, several dilutions of granulocytes were made in TC 199 medium and equal volumes (1.5 ml) were incubated with bacteria (1.5 ml, previously diluted in eugonic broth) for one hour at 37°C. The incubated suspensions were then centrifuged 10 minutes at 170 g

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* Pfizer Diagnostics Division, Pfizer Inc., New York, NY.

† Liquemine 10,000 μ per ml, Organon Lab, West Orange, NJ.

‡ Difco, Detroit, MI.
to remove granulocytes and phagocytized organisms. Supernatant fluid (3 ml) was placed in duplicate in the chambers of the Autobac I Cuvette and further incubated for 2.5 hours with agitation in the Autobac I Incubator/Shaker. At 30 minute intervals, the cuvets were removed and the density of bacterial growth in each chamber was measured. Voltage changes printed by the Autobac were utilized to determine the concentration of organisms represented by referring to the standard growth curves. Inhibition was calculated in percent by the following formula:

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\text{Inhibition (percent)} = \frac{\text{Original Concentration of Organisms} - \text{Concentration of Granulocyte Treated Organisms}}{\text{Original Concentration of Organisms}} \times 100
\]

In the preliminary experiments with this system, suspensions of \(E. coli\) were made in isotonic sodium chloride by adding fresh \(E. coli\) by bacteriologic loop to sterile saline until the density was such that the reading in the Autobac I nephelometer was exactly at the mid-point of the meter. Two ml of these suspensions were added to 18 ml of eugonic broth, and serial dilutions were made in eugonic broth. One ml of dilutions estimated to give concentrations of organisms between 10 and 1000 per ml was plated on blood agar plates. The agar plates were incubated at 37°C over night and the colonies were counted the next day. An average was determined from triplicate determinations on each of seven samples so prepared. The mean concentration of a 1 to 100,000 dilution was 344 with a standard deviation of 42 colonies per ml. Thus the mean concentration of starting \(E. coli\) suspensions was \(3.44 \pm 0.42 \times 10^6\) organisms per ml. Similar studies on \(S. aureus\) showed a mean concentration of \(2.25 \pm 0.31 \times 10^6\).

After incubation of \(E. coli\) with granulocytes, the cell pellet was examined for viable \(E. coli\). An additional step was done of lysing the granulocyte pellet in distilled water, centrifuging and placing the supernatant fluid in a cuvet with broth and measuring voltage change. This consistently gave results of little or no growth, indicating that few, if any, viable \(E. coli\) were lost in the pellet. Control tubes containing \(E. coli\) without granulocytes were also mixed with distilled water and allowed to incubate for 2.5 hours and no inhibition of \(E. coli\) growth was observed. Early work in this study was done using a cumulative, averaged, standard \(E. coli\) growth curve. Later experiments were carried out using a daily standard curve run with each experiment to reduce the effects of day to day variation in the measured growth characteristics of the microorganisms.

**Results**

The growth of the two bacteria studied, \(S. aureus\) and \(E. coli\), showed a lag phase and then a rapid development of density, the degree of which was related to the initial concentration of microorganisms (figures 1 a and b). The absolute density developed, as reflected in the change in voltage after 2.5 hours incubation in the Autobac I, was greater for \(E. coli\) than for \(S. aureus\). The relationship between the change in voltage was proportional to the log of the concentration of \(E. coli\), whereas a curvilinear relationship between voltage change and log concentration was observed with \(S. aureus\) (figures 2 a and b).

Inhibition of growth of \(E. coli\) by preincubation with granulocytes is proportional to the log of the granulocyte concentration (figure 3). \(S. aureus\) was virtually eliminated by incubation with granulocytes (figure 3) while measurable growth of \(E. coli\) allowed quantitative estimation.
of inhibition by granulocytes. Because of these differences (nonlinearity of voltage with density of growth and total inhibition of growth by granulocytes), work with S. aureus was discontinued and subsequent experiments were carried out using E. coli alone.

In early experiments, a single standard curve prepared from 17 separate runs was used to determine the number of organisms measured by a given voltage change. In later experiments, daily standard curves were prepared in an attempt to reduce day to day variability.

The results of E. coli growth inhibition by control granulocytes at a concentration of about 2 million per ml when determined by cumulative standard curve showed a mean of 78 percent ± 13 (S.D.) and by individual daily standard curve showed a mean of 79 percent ± 9 (S.D.). There was no difference in the absolute value of inhibition at this level of granulocyte concentration, but the standard deviation was less and the precision slightly better using the daily standard curve.

Examination of granulocyte preparations from different collection techniques revealed statistically significant differences in the ability of filtered granulocytes to kill E. coli when compared with centrifuged granulocytes. These differences existed when the granulocytes were tested after 24 hour storage as well as when they were tested in the fresh state (table I).

Discussion

Technically rapid and reproducible assays of granulocyte function are currently needed to evaluate methods of collection and storage of viable granulocytes for transfusion. Several studies have been published utilizing time consuming and poorly reproducible pour plate techniques for evaluating the bactericidal activity of granulocytes under various conditions of collection and storage.1,2,12,14 The effects of corticosteroids on bacteria and on granulocytes have been studied in this fashion.5,11,12

The present paper describes a rapid and reproducible technique for determining the bactericidal activity of granulocytes using the Autobac I nephelometer in a way similar to that for determining susceptibility of bacteria to antibiotics. Different concentrations of granulocytes collected and stored under different con-
The density of bacterial growth was expressed as the change in voltage (ΔV) in millivolts from the beginning of incubation to the final reading after 2.5 hours in the Autobac I. Conditions are added to the suspensions of microorganisms instead of antibiotic impregnated discs, and the inhibition of growth is determined nephelometrically.

Our studies indicate that centrifugation at 170 g does not remove bacteria from broth suspension in the absence of granulocytes, so this technique is suitable for separation of granulocytes and free microorganisms after exposure to granulocytes. Residual viable organisms can then be quantitated in the supernatant. Utilizing dilutions of known concentrations of bacteria, a standard curve can be prepared daily using the same organisms as those exposed to granulocytes to make most accurate and precise determinations of inhibitory action. The change in voltage (ΔV) for each sample tested can then be related to the number of organisms represented on the standard curve and the inhibitory effect can be calculated by a simple formula. Our experience shows that if microorganisms are prepared fresh from 18 to 24 hours growth on blood agar and handled the same way each run, a standard curve need not be run every day and the results of test samples from day to day will show a minimal increase in variation, which is not statistically significant.

There appears to be a difference in the relationship in ΔV and bacterial growth when standard S. aureus is compared with E. coli. ΔV showed a direct correlation with the log of the number of organisms with E. coli, but a curvilinear relationship with the log of the number of S. aureus. In addition, pour plate quantitation of standard bacterial suspensions showed 30 percent more E. coli than S. aureus were required to produce the same density reading on the Autobac I nephelometer (3.34 x 10⁷ E. coli to 2.25 x 10⁷ S. aureus). Whether or not these characteristics are in some way related to the physical shape of the organisms, one being a rod and the other a sphere, or to differing growth patterns is not clear at this time. E. coli proved to be most useful in showing variations in granulocyte bactericidal activity because some organisms remained to be grown after exposure to granulocytes, while virtually all staphylococci were killed by exposure to granulocytes even in the highest proportions of bacteria to granulocytes used in this study. E. coli, on the other hand, showed a progressive reduction in organisms as the concentration
of granulocytes to which they were exposed was increased.

In applying the method to evaluation of granulocytes collected by two methods, statistically significant differences could be demonstrated in bactericidal activity against *E. coli*. Granulocytes collected by continuous flow filtration were less effective in inhibiting growth of *E. coli* than were granulocytes collected by intermittent flow centrifugation. The differences in effectiveness of these two preparations was also demonstrable after 24 hours of storage at 4°C, although there was no major change in bactericidal activity in either type of granulocyte at 24 hours storage as has been demonstrated by others.3,14

The observation of differences between granulocytes prepared by filtration or centrifugation using this automated technique is in keeping with the reports of previous studies of bactericidal activity using pour plate techniques.8,12,14 There are now a number of studies which suggest that filtration derived granulocytes, while clearly more effective than no granulocytes, are not as effective as centrifugation derived granulocytes when measured in vitro. Other studies indicate chemotaxis of filtration derived granulocytes may also be reduced in comparison with centrifugation derived granulocytes.3,4,8,9,14

These considerations may become more important as newer techniques are developed for obtaining a better granulocyte product for the treatment of patients with neutropenia.

**Summary**

A method for determining the antibacterial effectiveness of granulocytes collected by different techniques using an automated nephelometer (Autobac I) is described. *E. coli* cultures showed a better correlation between concentration of granulocytes and bactericidal effect than *S. aureus* which were totally inhibited by granulocytes in all proportions used in this study. Using this technique, a statistically significant lower bactericidal activity was shown for filtration derived granulocytes as compared with intermittent centrifugation derived granulocytes.

**References**


