วารสารเทคนิคการแพทย์ เชียงใหม่
BULLETIN OF CHIANG MAI MEDICAL TECHNOLOGY
Vol. 6 No. 1
January 1973

PHAGOCYTOSIS AND KILLING FUNCTION (PKF) TEST OF NEUTROPHILS *

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Abstract

Phagocytosis and bactericidal activity are the outstanding features of polymorphonuclear neutrophils. Alteration of these activities has been found several conditions and generally related to the susceptibility to sepsis. The authors described their method for the determination of phagocytosis and bactericidal activity of neutrophils and illustrated its usefulness in detection of defective neutrophil functions in the patients with diabetes mellitus, typhoid fever and protein - calorie malnutrition.

INTRODUCTION

Many functions have been ascribed to polymorphonuclear neutrophils, but their outstanding feature is their capacity to phagocytize and degrade a variety of substances, paticularly bacteria. Functional defects of these cells are known to relate to the development of bacterial sepsis. Several techniques have been introduced for the study of the phagocytosis and bactericidal capacity of neutrophils with varying degrees of results even in normal individual. The reason for this may in part, be due to the difficulties and complexities of the given methodology plus the lack of standardization of techniques. We are describing our method which is rather simple, reproducible by which phagocytic and bacterial killing activities of neutrophils can be evaluated precisely in vitro.

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* Part of this work had been submitted to the Faculty of Medicine in partial fulfillment of the requirements for the B.Sc. degree (Med. Tech.).

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MATERIALS AND METHODS

Phagocytosis and bactericidal properties of intact leukocytes were determined by a modification of the method described by Hirsch and Strauss, (1) as modified by Quie, et al (2) and by Kauder et al. (3)

ISOLATION OF LEUKOCYTES

Leukocyte suspension were prepared by dextran sedimentation of heparinized venous blood. Ten ml. of freshly drawn bleod was collected into a sterile plastic syringe containing 200 units of sodium heparin and 2 ml. of sterile 6% dextran solution was added. After thorough mixing the syringe was allowed to stand upright on the plunger for 60 to 90 minutes at 4 °C. (or 30 to 60 minutes at 37 °C.). The supernatant plasma layer containing leukocytes was then harvested by bent needle technique and spun in sterile plastic tube for 5 minutes at 200 g. The cell button was washed twice with sterile Hank's balanced salt solution (HBSS), pH 7.2. After the last centrifugation the white cell concentration was determined by hemacytometer counting, smears were stained with Wright's stain and differential leukocyte count was performed. Cell suspension was then resuspend in sterile HBSS to give a polymorphonuclear neutrophils (PMN) concentration of 2×10^6 per ml. Ordinarily, over 95 % of PMN isolated by this technique are viable as shown by trypan blue or eosin-Y exclusion method and their functional integrity were intact as measured by latex particle phagocytosis.

PREPARATION OF BACTERIA

Stationary phase bacteria were used in the bactericidal assay system. Overnight (18 hours) broth culture (BHI. broth) of staphylococcus aureus, cosgulase positive, isolated from the patient of Chiang Mai University Hospital was centrifuged and washed twice with HBSS. A suspension of bacteria giving an optical density of 0.6 at 620 nm. in a Coleman Junior Spectrocolorimeter was prepared. This bacterial suspension was then further diluted and adjusted with HBSS to give approximately 2×10^7 bacteria per ml.

PREPARATION OF SERUM

Pooled normal human sera was frozen at -20 °C in one ml. aliquots and thawed immediatly before use to provide opsonins.

LEUKOCYTE-BACTERIA SUSPENSION

Phagocytosis studies were done in 12× 75 mm. sterile disposable plastic tube (Falcon) by adding 0.5 ml. of the PMN-rich suspension, 0.1 ml. of pooled sera, 0.1 ml. of the adjusted bacterial suspension and 0.3 ml. of HBSS. The mixture providing approximately two bacteria for each PMN in the medium containing 10% serum. The leukocytes bacteria mixture tubes tubes were then incubated at 37 °C in a temperature-controlled water-bath shaker set at 30 agitation/minutes.

Phagocytosis was determined by examination of smear from the leukocytes - bacteria mixture removed at interval and Wright-stained. The percentage of granulocytes containing ingested bacteria and the number of bacteria per cell (phagocytic index) were determined.

The leukocyte bactericidal activity was determined by the total number of viable bacteria in the mixture. Twenty five microliter aliquots of the incubated leukocyte-bacteria mixture were obtained from each culture tube at interval. This was diluted with 10 ml. sterile distilled water, mixed hard with vortex vibromixer for 30 seconds to facilitate osmotic disruption of the leukocytes. Viable bacteria were counted by diluting this suspension then mixed with the melted trypticase Soy Agar and using a standard pour - plate technique.

The number of viable intracellular bacteria was determined by adding 0.025 ml. aliquot of the leukocyte-bacterial mixture to 1.0 ml. HBSS containing 200 mcg. of Kanamycin sulfate then incubated for additional 30 minutes. This mixtuae was then washed twice with sterile HBSS to remove the kanamycin and extracellular bacteria. The cell button containing the intracellular bacteria was resuspended in 1 ml. sterile distilled water, mixed vigorously with vortex vibromixer for 60 seconds to facilitate osmotic disruption of leukocytes. The number of viable intraleukocytic bacteria was estimated by pour-plate technique as above.

RESULTS

The results of phagocytic studies performed on 8 individuals are illustrated in FIGURE I. In normal control individual, there is an increasing percent of phagocyts correlates well with the length of incubation time (exposure time of bacteria to PMN). The initial average phagocytic index is approximately 1.5 (number of bacteria ingested per neutrophil leukocyte). This value may be higher up to 3.0 or above in certain condition. Individual neutrophil may ingest as high as 15 to 20 bacteria into its cytoplasm. Two patients who has been given corticosteroid therapy for several months for their renal problems showed only minimal depression of phagocytosis in contrast to those with typhoid fever, protein - calorie malnutrition and diabetes.

Figure II illustrates nicely the normal pattern of bactericidal activity (capacity) of granulocytes of normal individuals. The other 3 patients with typhoid fever, protein - calorie malnutrition and diabetes showed a different pattern consisting of initial drop of the bacterial population due to the initial phagocytosis followed by a brief stationary period then increasing bacterial population reflecting the inability of their neutrophils to ingest and kill these bacteria.

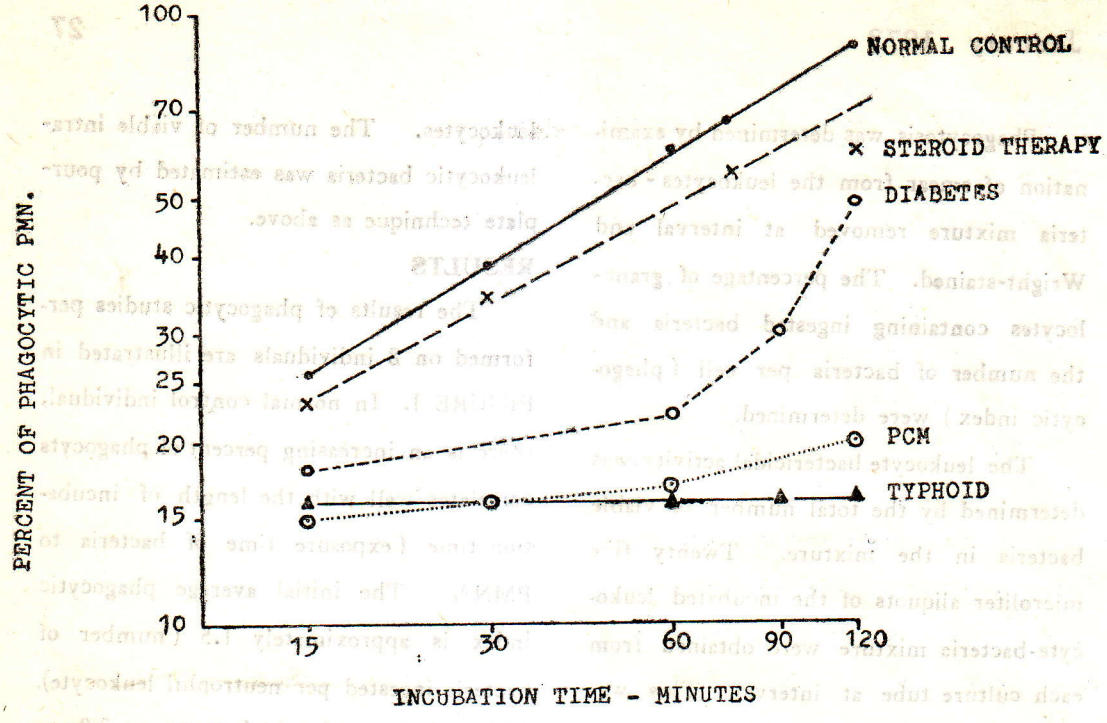

FIGURE I: The percentage of phagocytic PMN in normal individuals increased with the incubation time. Note the defective phagocytosis in other conditions.

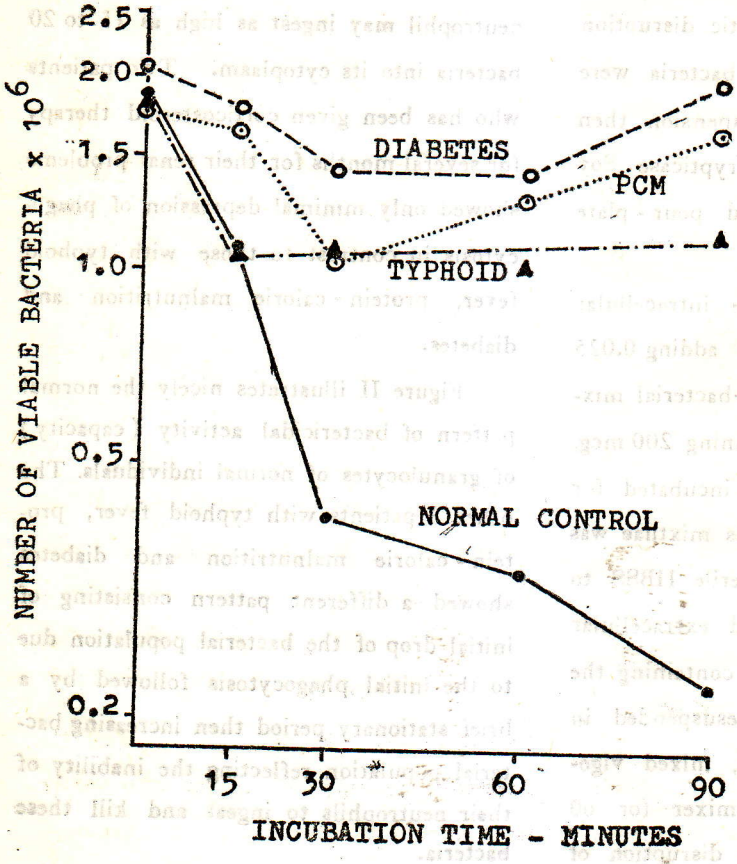

FIGURE II: The number of viable bacteria in the leukocyte-bacteria mixture is reduced by the bactericidal activity of normal PMN. Initial drop of viable bacteria is noted when the leukocytes with defective phagocytosis are used, then the viable bacteria population is elevated.

Kanamycin, in our hands, eliminate the possible extracellular bacteria contamination efficiently as well as the penicil lin-streptomycin combination advocated by others. It neither interfere with the degree of phagocytosis or intracellular viability of bacteria.

COMMENTS

The mechanisms of bacterial killing of normal PMN leukocytes are not completely understood. Phagocytosis bacteria is associated with the rupture of the leukocyte granules and the discharge of their contents into the phagocytic vacuole containing the ingested organisms. (4) Leukocytic granules contain a variety of antibacterial agents among which are lysozyme, a number of granular cationic proteins and myeloper- oxidase. There is a burst of leukocyte metabolic activity after phagocytosis that results in a sharp fall in pH in the vicinity of the ingested particle and in the generation of hydrogen peroxide by the cell (5) accompanied by the death of most organisms.

During the past few years several reports have appeared describing patients exhibiting deficiency in the phagocytic activity of blood PMN leukocytes. These can be separated into 2 general types of functional deficiencies :

1. Deficiency in bactericidal activity: Leugocytes of patients with chronic granulomatous diseas have been reported to contain normal amounts of lusozyme, peroxidase and phagocytin but they are unable to destroy the ingested microorganisms or to reduce the NBT to blue formazan during phagocytosis. It has been suggested that this may be due to the defective operation of cyanide insensitive NADH oxidase of the leukocytes. (6) The other examples of this type of defect is the impared killing of staphalococci, (7) myeloperoxidase deficiency (8) and Chadiak-Higashi syndrome. (9)

2. Deficiency of extracellular factors (and/or the opsonizing factors) including the opsonic defects described in sickle cell anemia (10), Miller syndrome (11), and Tuftsin deficiency (12). Opsonins are known to be either heat labile or heat stable, the latter being more efficient. The absence or diminution of adequate opsonization results in the failure of the phagocytic cell to engulf the infecting or target organism. The other examples of these deficiencies in certain components of the complements such as C 3, (13) C 5 (14) etc.

The possibility of varying amount of opsonizing activity in our system is eliminated by washing the leukocytes and adding the pooled fresh human sera. The other microorganism such as E. Coli, S. marcessen has been used sucessfully. Corticosteroid therapy in humans occasionally leads to increased susceptibility to bacterial

infections. Our results in 2 patients agreed with the others (15) that corticosteroids do not influence the normal phagocytic ability of PMN leukocytes, but they may interfere with intracellular killing activity (16) (17). Solberg and Hellum (18) studies 100 patients with bacterial infections had found that 32 % of these patients had reduced bactericidal activity. The reduced granulocyte function observed seemed to be the result of the infection rather than the cause. Some of their patients exhibited shift to the left of the neutrophil with reduced bactericidal activity similar to our patient with typhoid fever. It is important that the functional defects of PMN leukocytes of patients with typhoid, protein - calorie malnutrition and diabetes should be further investigated especially their relation to bacterial sepsis.

SUMMARY

The modified method for determination of phagocytosis and bactericidal activity of PMN leukocytes is described. By using this method it is possible to detect the functional defects of PMN leukocytes isolated from the patients with diabetes mellitus, protein - calorie malnutrition and typhoid fever.

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