THE EFFECTS OF SOME ANTIBIOTICS AND LIPOPOLYSACCHARIDE ON OXIDATIVE BURST AND PHAGOCYTOSIS OF NEUTROPHILS

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Dissertation submitted in fulfilment of Part III Master of Medicine (Microbiology) degree
University of Cape Town, January 1990

To Ursula

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ACKNOWLEDGEMENTS

First I would like to thank Dr Joseph Staneck for his idea to combine flow cytometry and antibiotics, as well as for his patience during the laborious experimental phases and his "green pen" correction exercises. Many thanks to professor Arderne Forder for his encouragement and support after my return to Cape Town.

Linda Trinkle, the flow cytometry expert, was always full of new ideas and suggestions and had to patiently endure the hour long repetition of experiments. I would like to thank Dr Lana Weckbach for her scientific counsel and for assistance in the production of figures. I wish to thank Naomi Hayes, Joyce Turner, Mary Anne McMillan and Linda Botha for their help in manuscript preparation.

CONTENTS

The Effects of Some Antibiotics and Lipopolysaccharide on Oxidative burst and Phagocytosis of neutrophils

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ABSTRACT

The determination of phagocytosis (P) and oxidative burst (OB) in unfractionated blood is a rapid and sensitive flow cytometric method for quantifying neutrophil activation, and was modified for single laser systems by using propidium iodide labeled Staphylococcus aureus (S.aureus) as a quantitative measure of phagocytosis and simultaneously the green fluorescence of oxidized 2'7' dichlorofluorescein diacetate was used to measure oxidative burst. Propidium-iodide labels dead organisms by intercalation with the DNA of the dead organism.

This assay was characterized with respect to the stimulatory activity of bacterial lipopolysaccharide (LPS) on OB and P and to determine the effects of lipopolysaccharide and different antibiotics on oxidative burst and phagocytosis by polymorphonuclear leukocytes (PMNL) using a FACS 440 flow cytometer.

Blood from healthy donors was pre-incubated with log doses of bacterial LPS (0.1 ng/ml - 1000 ng/ml) or sterile pyrogen-free saline at 37 ^{O}C from 0-120 minutes.

LPS increased both phagocytosis and oxidative burst in a dose-dependent manner (up to 62 and 121 percent respectively) at all time points tested, and this effect on P and OB could be detected even with no pre- incubation. This LPS - induced phagocytic activity could be blocked by the addition of polymyxin B (10 μg/ml) during pre-incubation. The priming effect of LPS was maximal at 45 minutes. P and OB were inhibited by pre-incubation with EDTA at doses greater than 1000 μg/ml (60 and 80 percent inhibition) respectively. These observations are consistent with the exquisite sensitivity of the neutrophil to endotoxin.

Blood from healthy subjects was subjected to different concentrations of antibiotics, or sterile pyrogenfree saline at 37 °C from 0 to 120 minutes. The antibiotics used were the following: Ceftriaxone, ciprofloxacin, clindamycin, doxycycline, enoxacin, imipenem, norfloxacin, pefloxacin, teicoplanin, tetracycline and vancomycin. Blood from healthy subjects was exposed to concentrations of the above antibiotics ranging from 0.1 to 200 μg/ml for 60 minutes. EDTA and bacterial LPS used as system controls demonstrated dose-dependent inhibition and increase respectively. Doxycycline showed inhibition of both parameters, while pefloxacin enhanced and tetracycline inhibited oxidative burst. The remaining antibiotics showed no dose-related modulation of either oxidative burst or phagocytosis.

The method described provides an environment that mimics physiological conditions; is a rapid and sensitive assay not requiring separation of white cells; and simultaneously measures two neutrophil functions. It can evaluate neutrophil response to immunomodulatory and chemotherapeutic agents in a physiological milieu. The necessity of using pyrogen - free reagents in any study of neutrophil function is re-emphasized.

KEYWORDS: Oxidative burst, phagocytosis, lipopolysaccharide, flow cytometry, unfractionated blood, endotoxin, polymorphonuclear leukocytes, neutrophils, PMNL.

INTRODUCTION

Polymorphonuclear leukocytes (PMNL), particularly neutrophils, which are part of the non-specific defense mechanisms of the human host, engulf bacteria by a process called phagocytosis and play a key role in non-specific host defense against bacterial invasion.

Human neutrophils have been implicated as mediators of tissue-destructive events in inflammatory diseases ranging from rheumatoid arthritis and myocardial reperfusion syndromes, blistering skin disorders, and ulcerative colitis. In these diseases, important parts of the pathogenesis of these diseases is being linked to the neutrophil's capacity to release a complex assortment of agents that may destroy normal cells as well as connective tissues. As the knowledge of the neutrophil's potential of tissue damage increases, so does the need of therapeutic agents which may downregulate these unwanted effects (Weiss 1989).

The initial encounter between a bacterium and the PMNL results in ingestion or engulfment of the microorganism through phagocytosis, a process mediated by serum factors. Various intracellular processes are set in motion after this event which usually end in the destruction of the invading bacteria. Subsequent digestion and destruction of bacteria are brought about by a series of intracellular events including degranulation (lysosomal fusion followed by release of lysosomal contents), and the manufacture of cytotoxic oxidants within the PMNL, events collectively referred to as "oxidative burst" (Babior 1982).

As the leukocyte engages bacteria or other ingestible particles, membrane stimulation results in a dramatic shift of oxygen metabolism (Babior 1982). Concurrent with a marked increase in oxygen consumption, hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) are produced. These oxidants are bactericidal and are in part responsible for the intracellular killing and subsequent destruction of the ingested microorganism.

The activity of phagocytosis and related functions can be affected by numerous factors as generalized as patient age, underlying illness, trauma, and surgery or more specifically, the presence of specific opsonizing antibody or particular medications. (Bass 1984, Bassoe 1984, Nagel 1986, Seneca 1986). It therefore has long been of interest to monitor phagocytic activities by in vitro testing. Traditionally, assessment of phagocytosis has been carried out by manual microscopic examination of ingested bacteria stained with conventional or fluorescent stains (Fig.1); the determination of total cell associated particles by using antigen-treated dye-containing oil droplets or the enumeration of either ingested or non-ingested bacteria by colony counts (Bassoe 1981, Dunn 1984). Alternatively, the detection of particle uptake has been monitored through radiolabelling or electronic particle counting techniques. Chemiluminescence has been used as an indicator of OB during phagocytosis. These methods vary in accuracy, demand preparative steps to isolate PMNL and are often tedious and time-consuming (Wilson 1985).

Numerous flow cytometric methods of quantifying neutrophil phagocytosis have been described (Bassoe 1983, 1984, 1985) in which measurement of fluorescence associated with PMNL following ingestion of fluorescent labelled particles serves as an indicator for phagocytic activity.

Briefly, using a flow cytometer (Fig. 2), leukocytes are introduced into the center of a rapidly moving fluid stream and are presented in single file, at uniform speed, to a measurement station. Typically light sources present a beam that is subsequently scattered at different angles, analysis of which provides information on cell sizes, shape, viability, density and morphology. Therefore, cellular elements of a certain type, (in this case PMNL) can be selectively chosen for analysis (Fig. 3). The use of fluorescent dyes and the subsequent analysis of fluorescence provides an even more powerful analytical tool (Quirke 1986). The number of cells available for analysis by flow cytometry greatly exceeds that practical by a human observer using manual methods for the measurement of phagocytosis and does so with greater accuracy, reliability and with less variation (Dunn 1981).

These flow cytometric methods have been compared with manual microscopic methods of quantifying phagocytosis and found to be comparable in results, but significantly faster and more accurate (Dunn 1981). It has now been accepted as a recognized method of assessing neutrophil function and is being used in many routine diagnostic laboratories.

More recently the technology of flow cytometry has enabled the assessment of both OB and P using isolated leukocytes (Bassoe 1984, Bass 1984, Dunn 1981). These assays for the simultaneous measurement of phagocytosis and oxidative burst permit the observation and quantification of oxidative burst in response to a physiological trigger.

Oxidative burst is quantified by measuring the fluorescent reaction product of 2'7' dichlorofluorescein diacetate (DCFH-DA) and hydrogen peroxide. The non-polar, non-fluorescent compound 2'7' DCFH-DA is loaded into PMNL by passive membrane diffusion. Once inside the cell, the dye is deacetylated by non-specific esterase to form the polar, non-fluorescent dichlorofluorescein (DCFH). Reactive oxygen species released after phagocytosis oxidize the DCFH to form the highly fluorescent dichlorofluorescein (DCF) (Bass 1984, Keston 1965, Szedja 1984).

Trinkle and Wellhausen (1987) recently combined the above mentioned principles and described a flow cytometric assay using unfractionated (whole) blood that allows for the simultaneous assessment of P and OB through measurement of Texas Red stained-bacteria and the quantification of the fluorescent form of dichlorofluorescein diacetate resulting from activity of PMNL oxidative enzymes. They demonstrated the enhancement of these neutrophil activities in healthy donors in response to the addition of 10 μg/ml of LPS as well as in patients with bacterial infection.

Many preparative methods for neutrophil isolation have been shown to activate neutrophils due to contaminating amounts of LPS in culture media animal sera, albumin and Ficoll solutions (Davis 1980, Dunn 1981, Guthrie 1984).

Lipopolysaccharide (LPS), or endotoxin, is a major component of the outer membrane of Gram-negative bacteria. LPS is unique to the outer membrane, its lipid terminus providing a large part of the lipid phase of the outer bilayer of the membrane, while its long polysaccharide side chains provide surface antigens. Even in the absence of viable organisms, LPS has been associated with numerous diseases (e.g. disseminated intravascular coagulation, endotoxaemia following from Gram-negative septicaemia, etc.) resulting in injuries to lung and kidney and other vital systems (Levin 1970, Smedly 1986). Endotoxin causes many of the abnormalities associated with death from Gram-negative bacteraemia. Antibiotics do not reverse evolving shock, but are required to clear bacteraemia. Endotoxin free in the blood activates mediators of cell damage until natural clearance - and detoxification mechanisms are able to handle the load (Ziegler 1988). The administration of endotoxin to normal subjects causes a depression of left ventricular function independent of changes in the left ventricular volume or vascular resistance. This suggests that endotoxin is a major mediator of the cardiovascular dysfunction of septic shock (Suffredini 1989). Available clinical data suggest that during septic shock organisms with vastly different structural and molecular compositions can activate a common pathway leading to qualitatively similar severe cardiovascular dysfunction (Natanson 1989). Endotoxin mediated clinical problems often have high morbidity and mortality rates. In the U.S. alone, 90 000 deaths occur annually due to Gram-negative sepsis.

Numerous studies in experimental animals have shown that isolated endotoxin could virtually reproduce all of the clinical manifestations observed in patients with Gram-negative sepsis (Shenep 1984).

According to Levin (1970), during endotoxaemia, the concentration of LPS in plasma has been measured to be between 0.5 and 5 ng/ml. The host responds to the presence of LPS in a variety of ways. LPS has been shown to enhance both P and OB of neutrophils in vitro (Trinkle 1987) and enhances neutrophil emigration in vivo in rabbits (Cybulsky 1988). In vitro, LPS at concentration of 1-10 ng/ml "primes" neutrophils enabling them to respond to chemotactic factors with an enhanced release of oxygen radicals and lysosomal enzymes toxic to the ingested organisms (Guthrie 1984). LPS also stimulated neutrophil adherence to plastic (Dahinden 1983) and to human endothelial cells (Smedly 1986). This activation of the inflammatory process is a double-edged sword as these seemingly advantageous properties also cause host tissue damage.

The mechanism of neutrophil-mediated host injury is not entirely clear but has been reported to be due to the effects of neutrophil release of hydrogen peroxide of superoxide anions and lysozyme secretion on host target cells (Smedly 1986 ; Wilson

1982). Despite these detrimental effects, the selective use of modified "non-toxic" endotoxin molecules (monophosphosphoryl lipid A), has been proposed to stimulate the body's natural defence mechanisms (Ribi 1984). LPS can be extracted from intact cells and separated into a lipid A component which retains toxicity and a polysaccharide portion which has antigenic specificity (Ribi 1984).

The cationic antibiotic polymyxin B binds to the lipid A portion of the LPS molecule (Morrison 1976, Wood 1978) and has been shown to block LPS-induced activation of human leukocytes in vitro (Lachman 1983).

Employing a modification of the Trinkle assay, we have described a rapid and inexpensive method for measuring the effects of antibiotics and LPS on phagocytic and oxidative burst of PMNL. Additionally, EDTA and heparin, two commonly used anticoagulants, were investigated for their impact on these neutrophil function tests.

The described technique utilizes whole blood thereby avoiding the necessity of PMNL separation and attempts to provide a simulation of an in-vivo environment. The method also minimizes mechanical or pyrogenic perturbation of the leukocytes which may lead to erroneous results (Wood 1978, Fearon 1983, Haslett 1985).

MATERIALS AND METHODS

Blood donors

Fresh unfractionated blood from 24 healthy human laboratory workers was obtained by a clean venipuncture with pyrogen - free needles into sterile 10 ml acid-citrate-dextrose Vacutainer tubes (ACD) (Becton Dickinson Vacutainer Systems, Rutherford, NJ). These tubes are commonly used for cell isolation procedures.

It was very important to adhere to a good phlebotomy protocol which included a short application time of the tourniquet, a good clean venipuncture with no difficulty of penetration of the venous wall and with minimisation of manipulations to obtain free-flowing blood. A difficult phlebotomy results in activation of the leucocytes. As the assay is dependent on the relative availability of leukocytes, each donor's white blood cell status was determined by analysis of a sample of donor blood in an EDTA tube on a Coulter counter. This procedure excluded subjects with granulocytopenia or granulocytosis.

In experiments comparing ACD and heparin, blood from each volunteer was collected first into a complete blood count tube (CBC) containing Potassium EDTA, followed by collection of blood into an ACD or heparin tube. All assays were performed within eight hours after obtaining the blood at the latest, but usually within 3 to 4 hours.

Reagents:Preparation of Bacteria and DCF

Phagocytosis was quantitated using a commercially prepared fixed Staphylococcus aureus (trade name Pansorbin 10% wt/vol, Calbiochem, San Diego, CA) stained with propidium iodide (PI; Calbiochem, San Diego, CA). Equal volumes of Pansorbin and

PI (100 μg/ml) were mixed and allowed to stand for 30 minutes at room temperature. The labelled bacteria were washed twice and resuspended in Hanks Balanced Salt Solution (HBSS, (Gibco, Grand Island, NY) to achieve a final concentration of 5% w/v for use in the phagocytosis assay.

Aliquots of 5 mM 2'7' dichlorofluorescein diacetate (DCFH-DA, Eastman Kodak Co., Rochester, NY) were stored in absolute ethanol at -20 $^{\circ}$ C until diluted ten-fold in sterile HBSS for use in the assay. 2'7' dichlorofluorescein diacetate was dissolved in absolute ethanol. Different concentrations were evaluated, but 50 μM DCF was found most useful and became the standard testing concentration. All reagents were prepared with pyrogen-free saline or water, and all glassware used was sterilized.

Reagents: Red cell Lysis reagent

The red blood cell lysis agent consisted of 0.15 M ammonium chloride (Fisher, Fair Lawn, NJ), 0.01 M potassium bicarbonate, and 0.1 mM tetrasodium EDTA (Matheson, Coleman and Bell, Norwood, OH), dissolved in pyrogen - free saline (Abbott Laboratories, Chicago, IL).

Reagents: Antibiotics

The following antibiotics were obtained in reference powder and were dissolved in sterile saline in most cases: Doxycycline (Pfizer, New York, NY), tetracycline (American Cyanamid Co., Pearl River, NY), ciprofloxacin (Miles Pharmaceuticals, West Haven, CT), ceftriaxone (Hoffman La Roche, Inc., Nutley, NY), norfloxacin (Merck, Sharp and Dohme, West Point, PA), clindamycin (Upjohn, Kalamazoo, MI), teicoplanin (Merrell - Dow, Cincinnati, OH), imipenem (Merck, Sharp and Dohme, West Point, PA), vancomycin (Eli Lilly and Company, Indianapolis, IN), enoxacin

(Warner Lambert, Morris Plains, NJ), and pefloxacin (Roger Bellon, 159 Avenue A. Peretti, 92200 Neuilly Sur Seine).

Certain antibiotics were dissolved in alternate solvents as directed by the manufacturer. For example, norfloxacin was reconstituted in 0.1 N NaOH, imipenem in pH 7 phosphate buffer, and enoxacin in 5 % ethanol and 0.1N NaOH. For each antibiotic solution tested, a control was prepared which contained only the solvent used for that antibiotic solution.

The antibiotics were made up in stock solution and frozen at 70^{0} C in electron beam sterilized polypropylene tubes (Sarstedt, Nürnbrecht, West Germany), with the exception of imipenem which was prepared fresh for each experiment. Ciprofloxacin was stored in borosilicate glass vials. Each antibiotic was tested with the PMNs of at least three different donors. When major differences were observed in activity between control cells and cells exposed to antimicrobials, additional experiments were performed for confirmation.

LPS, Polymyxin and EDTA

Escherichia coli O55 : B5 Lipopolysaccharide (LPS) (Lots 3120-25-0 and 3923-25-9, Difco, Detroit, MI); polymyxin B (Sigma) and tetrasodium EDTA (Matheson, Coleman and Bell, Norwood, OH) were dissolved and diluted in sterile pyrogen - free saline as fiftyfold stock solutions. Aliquots of these solutions were frozen at -20^{0} C.

Unfractionated Blood Phagocytosis and Oxidative Burst Assay

A unfractionated blood assay described by Trinkle and Wellhausen has been modified for use with single laser excitation (Trinkle 1987). Each frozen or freshly -made antibiotic stock solution as fiftyfold stock concentration was diluted appropriately. Ten microliters of the appropriate concentration of test reagent (sterile pyrogen- free saline, antibiotic, LPS or antibiotic solvent) were added to 440 μl of unfractionated blood in disposable tissue culture sterile polypropylene tubes (12 x 75 mm, individually wrapped).

Stationary incubation at 37^{0} C in a water bath was carried out for 60 minutes. The addition of 50 μl of 500 μM DCFH-DA solution to the polypropylene tubes of pre-incubated blood followed by incubation for 10 minutes in a 37^{0} C shaking water bath at 112 cycles / min allowed for transport of DCFH-DA into the leukocytes. A 50 μl aliquot of blood was then removed for time zero (t_0 baseline) analysis of dye loading and endogenous peroxide levels prior to the phagocytosis related oxidative burst. Phagocytosis was initiated by the addition of 50 μl PI labelled S. aureus. Agitation of the tubes in the water bath was maintained throughout the experiment. At timed intervals of 5 (t_5) and 10 minutes (t_{10}) after addition of the bacteria, two additional 50 μl aliquots were taken using pyrogen - free pipettes. These aliquots were exposed to red blood lysis reagent for 5 minutes.

Each sample was placed in 1.5 ml of red blood lysis reagent consisting of 0.15 M ammonium chloride, 0.01 M potassium bicarbonate and 0.1 mM tetrasodium EDTA, mixed thoroughly and allowed to incubate five minutes at ambient temperature. Following RBC lysis, the WBC were centrifuged for 2 min at 500 g, the sample was washed with 1 ml HBSS, re - centrifuged and resuspended in 200 μl of HBSS. Each sample was placed on ice approximately 12-15 minutes after sampling and analyzed within 1 hour.Trypan blue quenches extracellular fluorescence and was initially used to distinguish between adherent (non- ingested) and ingested particles (Bjerknes 1984, Hed 1987). No marked differences in fluorescent activity were found and further samples were analyzed without trypan blue.

Kinetic Study of LPS Activation

LPS was added to unfractionated blood as described and allowed to incubate in a standing water bath, 37 ^{0} C for 0 (zero time), 15, 30, 45, 60, 90 or 120 minutes prior to the addition of DCFH-DA. The blood was sampled prior to and 10 minutes after the addition of the S. aureus and processed as described above.

Blocking of LPS effects by Polymyxin B

When polymyxin was used to block LPS effects, the polymyxin dose was added immediately prior to the LPS dose. LPS was tested at 0.1 ng, 1 ng, 10 ng, 100 ng and 1000 ng. Polymyxin B (10 μg/ml) was tested against the four highest LPS doses. Sodium EDTA was tested at 20, 200, 400, 1000 and 2000 μg/ml.

Flow Cytometry

Cells were analyzed by a FACS 440 flow cytometer (Becton Dickinson, Mountain View, CA) with Consort 30 data handling system. The 488 nm line from an argon laser operating at 400 mW was used to excite both the DCF and PI. DCF-related log green fluorescence was collected using a 530/15 bandpass filter (Becton Dickinson) and recorded as a measure of oxidative burst. PI-related log red fluorescence was collected using a 580 long pass filter (Ditric Optics) and recorded as a measure of phagocytosis. Standardization was performed daily by means of FITC and PI fluorescent beads (Flow Cytometry Standards Corp., Research Triangle Park, NC). Electronic compensation was used to eliminate fluorescence due to spectral overlap of the fluorochromes. Polymorphonuclear cells were selected for on the basis of forward and right angle light scattering properties (Fig. 3). Each sample consisted of 3000 PMNL and these cells were counted and the mean channel fluorescence recorded.

RESULTS

The results of assays with control cell populations e.g. antibiotic-free saline were expressed as follows: A ratio of measured mean fluorescence of non-exposed cells (i.e. only exposed to sterile saline) relative to the exposed control cells (i.e. exposed to an antibiotic or LPS) was calculated and expressed as a percentage (%). This meant e.g. that the control cell population fluorescence was represented by 100 %. Concentrations of LPS and antibiotics described were final concentrations in the unfractionated blood. Concentrations used were based on previously published values shown to influence neutrophil function parameters (Daschner 1985, Duncker 1986) or on clinically relevant levels.

Comparison of Heparin and ACD Anticoagulants

Unfractionated blood was collected in either ACD or heparin Vacutainer tubes and assayed for phagocytic and oxidative burst activity as described without pre - incubation. In every case using blood from four donors, DCF-related green fluorescence of the heparinized blood sample was 50-75% lower than the paired ACD blood sample when measured prior to the addition of the bacteria and also at 10 minutes after the addition of the stimulus. However, the phagocytosis-related red fluorescence was slightly higher in the heparinized blood samples (up by 10%) when compared to the ACD blood. Subsequently, only ACD blood was used in all further experiments.

Kinetic Study of Effects of LPS

Unfractionated blood from four individuals was tested to determine the rapidity with which the neutrophil responds to endotoxin. Blood was pre-incubated as described with 100 ng/ml or 1000 ng/ml LPS or sterile saline for up to 2 hours prior to initiation of the neutrophil function tests. The priming effect of LPS in the t_0 samples showed consistent activation as measured by increased DCF fluorescence with 45 minutes of pre-incubation with LPS at the above doses and continued for the remainder of the pre-incubation time points (Fig. 4a). Effects of LPS on oxidative burst were detected in cells at all pre-incubation time points, including zero time incubation samples for which the OB assay was initiated immediately upon addition of the LPS. However, a consistent effect on all phagocytosis samples was not observed until after 15 minutes pre-incubation with LPS (Fig. 4b). Phagocytosis seemed to be maximal at 45 minutes (Fig. 4c). The amount of phagocytosis per cell was relatively constant for each group from 30 minutes to two hours, but the oxidative burst activity was diminished with increasing time after the 45-minute peak.

LPS Dose Response

In order to investigate whether this method would demonstrate effects of endotoxin on PMNL at levels of LPS consistent with those described in the serum of patients with Gram-negative bacteraemia (Levin 1970), fresh unfractionated blood from eight individuals was incubated for 60 minutes at 37^{0} C with sterile saline or LPS at concentrations ranging from 0.1 ng - 1000 ng/ml. (Fig. 5). An increase in OB at concentrations of 1 ng/ml and greater after the 10-minute dye- loading step was apparent whether tested immediately prior to the initiation of phagocytosis or at 5 or 10 minutes after addition of bacteria. This priming effect of LPS was somewhat dose-dependent, as demonstrated by increase in P and OB at increasing concentrati2ons of between 0.1 and 100 ng LPS. The 1000 ng/ml LPS dose showed no additional, or in some cases less of a priming effect than did lower LPS concentrations. All individuals tested showed marked enhancement of both P and OB in both the 5 and 10 minute samples (mean activity up to 149% of control cells for P and 178 % of control cells for OB) at LPS doses of 1 ng/ml or greater. However, variable sensitivity to endotoxin at the 0.1 ng/ml dose was noted among individual subjects. Six of eight individuals showed enhanced phagocytosis and to a lesser degree increased oxidative burst at this low dose, while only four out of eight showed a primary effect of LPS in the t_0 sample with 1 ng/ml LPS.

Blocking of LPS by Polymyxin B

Polymyxin B binds to the lipid A region of the LPS molecule and has been shown to prevent LPS induced activation of leukocytes when co-incubated with LPS in the absence of serum (Morrison 1976, Wood 1978). To test whether this effect could be demonstrated in a unfractionated blood system, polymyxin B (10 μg/ml) was added to blood prior to the addition of LPS. It was found that when the ratio (w/w) of polymyxin to LPS was 1,000:1 or greater, inhibition of LPS activity could be demonstrated (Fig. 6). At 100:1, the values for phagocytosis and oxidative burst approached the level of a LPS-free control.

EDTA Effects on Unfractionated Blood Phagocytosis

To determine whether phagocytosis and oxidative burst could be inhibited in this assay system, cells were pre-incubated for 60 minutes with Na EDTA at 20 μg, 200 μg, 400 μg, 1000 μg and 2000 μg/ml as described. Inhibition of oxidative burst and phagocytosis was seen at 1000 μg/ml and above. Phagocytosis was inhibited at the two higher doses but was unaffected at 400 μg/ml and lower (Fig.7). Oxidative burst was totally inhibited at the two higher doses22 and was reduced in some, but not all individuals at the lower doses.

Antibiotics

For the antibiotic side of the experiments, LPS, a potent stimulator of P and OB, and EDTA, an inhibitor of phagocytosis at concentrations above 1000 ug/ml, were used to assess the validity of the flow cytometric assay at concentrations described above.

Twenty four different blood donors were used for the antibiotics study. Eleven different antibiotics were tested at different concentrations. A total of 681 samples including controls were tested-this means the total number of cells counted amounted to a total of 2 043 000 cells. Unfractionated blood exposed to antibiotic was sampled immediately before the addition of S. aureus and 5 to 10 minutes after addition. Since LPS stimulation experiments consistently gave the highest readings at 5 minutes under conditions of the assay, the antibiotic data presented are those calculated from the 5 minute values (Tables 1 and 2). Values obtained at 10 minutes (not shown) varied only slightly from those observed at the 5 minute reading. The mean activity of cells with the majority of antibiotics tested fell within 10 % of the activity of the respective antibiotic-free control cells, i.e. between 90 and 110 %. As noted in Tables 1 and 2, pefloxacin for example at concentrations of 200 ug/ml had a mean % activity of 110%, while doxycycline, norfloxacin and tetracycline had reduced OB activity to less than 90 % of the activity of control cells. Doxycycline and tetracycline showed reduced phagocytosis activity to less that 90 % of antibiotic-free control cells.

The antibiotic with the most prominent inhibitory effect was doxycycline. At 200 μg/ml, OB was reduced to a mean of 10 % of the control activity (range 10% and 22%). Phagocytosis was reduced to a mean of 25% of control activity (range 14-32%). With increasing concentrations of doxycycline, a dose-related modulation of response was seen with both OB and phagocytosis (Fig. 8).

Results with tetracycline were less dramatic than those obtained with doxycycline. Oxidative burst for tetracycline was reduced to 77% of control cell activity at 200 μg/ml. One donor's cells showed very slight inhibition of OB and phagocytosis when exposed to tetracycline at 200 μg/ml. Upon inquiry she was found to have been taking tetracycline for many years. This donor was therefore excluded from subsequent data analysis in that tetracycline may have been present in the control cells. At lower, clinically relevant concentrations of tetracycline, the only inhibitory effect on OB was seen at 10 μg/ml with a mean of 86% of control activity. Phagocytosis was not consistently affected at any of the tetracycline concentrations tested.

Pefloxacin-exposed cells showed a dose-dependent increase of OB activity, while phagocytosis was not enhanced. At 200 μg/ml, the mean OB value obtained was 138% of control activity (range 95-177%), at 100 μg/ml 123% (range 101-141%), and at 10 μg/ml 117% (range 104-125%) (Fig. 8).

Occasionally, a mean percentage value for experiments at a certain drug concentration was noted to vary more than 10 % from the control activity for one or another parameter, e.g. 200 μg/ml norfloxacin (Table 1).

However, none of the other eight antibiotics tested appeared to demonstrate dose-related modulatory effects. Therefore, it was concluded that these drugs had no or at least marginal influence on OB or phagocytosis.

DISCUSSION

Various methods are available for assessing phagocytosis by PMNs. Some of these techniques are cumbersome and tedious, and often do not reflect the physiological condition. The assay described here results in simultaneous measurement of two neutrophil function tests, i.e. phagocytosis and oxidative burst in unfractionated blood, and is relatively easy to perform should flow cytometry facilities be available. Compared to other types of manual assays, particularly those involving microscopy and colony counting, the flow cytometric assay has a greatly reduced labor component and is therefore relatively inexpensive. It is imperative that pyrogen - free equipment and reagents be used. The inadvertent activation of oxidative burst by even picogram amounts of LPS may result in misinterpretation of effects. Some non-reagent grade substance used early in the developmental stages of this study resulted in substantial activation of oxidative burst. Additionally, heparinized blood should not be used as it proved less suitable in terms of dye loading.

We have modified a unfractionated blood method for the simultaneous quantification of phagocytosis and oxidative burst by neutrophils for use with a single-laser flow cytometer by using the DNA-intercalating dye, PI, to label fixed S. aureus and 2' 7' dichlorofluorescein as a fluorescent indicator of oxidative burst. Flow cytometric analysis of 22unfractionated blood preparations allows neutrophils to be "isolated" using the light scattering properties of these cells rather than mechanical, time-consuming gradient techniques which have been shown to alter the shape of cells, reduce chemotaxis and 0_2^- release as well as increase lysosomal enzyme release (Haslett 1985). Fearon and Collins (1983) demonstrated that an eight-fold increase in the expression of C3b receptors on neutrophils could be induced by normal preparative centrifugation procedures in which the temperature is lowered to 4^{0} C and then raised again. In addition to mechanical effects on the cells, isolation procedures require extremely stringent measures to avoid endotoxin contamination of the cell preparation. Haslett (1985) found contaminating LPS concentrations between 1 and 100 ng/ml in commercially prepared reagents for cell isolation.

A unfractionated blood system is ideal for examining the effects of various agents on leukocytes in that it avoids these potentially activating procedures and allows the examination of cellular function in a near physiological milieu. We found that by incorporating only pyrogen-free reagents, we were able to detect effects of sub nanogram levels of LPS on P and OB. Others have described similar enhancement of other neutrophil activities by ultra low levels of endotoxin (Guthrie 1984, Smedly 1986).

The influence of antibiotic therapy on non-specific defense mechanisms has many facets and has been evaluated from many different viewpoints in the literature. The concept of antibiotics depressing or enhancing host defense has been widely published (Daschner 1985, Seneca 1986, Forsgren 1984). The beneficial effects of antibiotics for the host reside in the impairment or destruction of the microbial invader. However, the immunomodulating role of antibiotics is not clearly defined. Ideally, an antibiotic should enhance or at least not prove to be inhibitory to the host defense mechanism. According to Milatovic (1983), the interactions between antibiotics and the phagocytosis process can be arranged in four major categories:

1) influence of antibiotics on chemotaxis;
2) direct action of antibiotics on the phagocytic cell functions;
3) uptake and / or killing of bacteria altered by previous contact with antibiotics;
4) intracellular penetration of the antibiotics and intracellular activity against phagocytosed bacteria.

The method described here evaluated only the interactions of category 2. Various other in vitro assays have been described which measure specific stages of this complex process; however, comparison of results is difficult since differences in experimental circumstances could influence the actual in vitro measurement. Factors such as the addition of serum and the separation of the white blo2222od cells may cause membrane structural changes and may alter in vitro functionality (Fearon 1983).

In our flow cytometric assay system, a marked reduction of both oxidative burst and phagocytosis after pre-exposure of PMNs to doxycycline was observed. Other studies have reported similar findings. Duncker (1986) showed suppression of chemiluminescence (CL) in vitro by doxycycline and tetracycline. These in vitro results correlate well with ours, despite the fact that CL is only a by-product of OB. Siegel and Remington (1982) showed significant inhibition of CL of PMNs by tetracycline and doxycycline. Geisen et al. (1986) also showed a reduction of CL and phagocytosis with doxycycline at 8 μg/ml after pre-incubation of diluted unfractionated blood for 10 minutes. Forsgren et al. (1984) looked at parameters of chemotaxis and phagocytosis in human PMNL pre-exposed to a range of antibiotics. Doxycycline was found to have a significant depressive effect on above parameters. Gnarpe (1974) found that when leukocytes were pre-exposed to doxycycline, the bacteria were found to be adherent to the granulocyte surface rather than ingested when observed by scanning electron microscopy.

Chemiluminescence was almost totally extinguished (Belsheim 1983) after FITC stained yeast cells were opsonized and mixed with granulocyte suspensions which had been pre-incubated with various concentrations or doxycycline. In other experiments (Gnarpe 1974), the number of phagocytosing cells was reduced to less than 50% of the normal when doxycycline was present at 10 μg/ml. Tetracyclines generally inhibit leukocyte chemotaxis, usually at 2.5-1000 μg/ml (Daschner 1985, Forsgren 1974, Grec 1984). The clinical significance of these findings is unknown. On the other hand, leukocytes of donors who had been exposed to oral doxycycline showed no change in ingestive capability compared to controls (Melby 1981) and no effect on monocyte phagocytosis (Athlin 1984, Forsgren 1984).

Few studies have demonstrated enhanced phagocytosis by antibiotics. Ceftriaxone, enoxacin and norfloxacin have been reported to enhance CL (Duncker 1986). It is interesting to note that these drugs require unusual solvents and the possibility of contamination of reagents with stimulatory substances cannot be excluded.

One curious finding in our study is the marked dose-dependent modulation of oxidative burst independent of an effect on phagocytosis by pefloxacin (Fig. 8). Pefloxacin's effect on phagocytic function of rat macrophages and PMNs was studied by Desnottes et al. (1986). They found both 1 and 100 μg/ml pefloxacin significantly increased the phagocytic capacity and activity of PMNL. This discrepancy with our phagocytosis data could possible be due to the differences in the assay systems, i.e. human unfractionated blood vs. isolated rat PMNL or due to an effect of pefloxacin on the live yeast during the 2 hour incubation with PMNL in Desnottes' system. Boogaerts et al. (1986) studied the effects of the new quinolones on PMNL function by chemiluminescence, myeloperoxidase, superoxide production, phagocytosis and chemotaxis. No differences for these parameters were found for ciprofloxacin, norfloxacin, pefloxacin and ofloxacin, except for a 5% stimulation of phagocytosis with 10 μg/ml norfloxacin a 10 μg/ml pefloxacin. Our data do not confirm this finding (Table 1). In agreement with our observations, no direct effect on phagocytosis by human granulocytes was found with the quinolone derivatives ciprofloxacin and norfloxacin by Forsgren (1985). However, they observed enhanced killing of S. aureus by PMNL exposed to sub-inhibiting levels of ciprofloxacin.

In our assay, neither teicoplanin nor vancomycin showed a dose-modulated response on the leukocyte parameters measured. Fietta et al. (1986) showed that neither teicoplanin nor vancomycin interfered with phagocytosis. However, teicoplanin but not vancomycin in that report enhanced intracellular killing by neutrophils from normal donors.

Numerous conflicting results on the direct effects of antibiotics on leukocytes have been reported. It appears from our findings that very few antibiotics at clinically relevant concentrations directly influence either the respiratory burst or phagocytosis by PMNL.

An increase in unfractionated blood phagocytosis in all individuals tested, was seen with concentrations as low as 1 ng/ml LPS. In many individuals tested, 100 pg/ml LPS could prime the neutrophils after 1 hour exposure at 37 ^{o}C. In our time study in which the effects of the length of PMNL exposure to LPS were examined, we found that even when LPS was added immediately prior to the addition of 2'7' DCFH-DA, we could measure an increase in OB. However, we did not see a consistent stimulatory effect on the t_o sample until after 45 min incubation with LPS (Fig. 4a). One could speculate that this delay in activation of the neutrophil could indicate that this t_o priming may not be due to a direct effect of LPS on neutrophils, but may be an interleukin-1 (IL-1) mediated response. Cybulsky et al. (1988), in a study examining effects of LPS, IL-1 and tumor necrosis factor α (TNF) on neutrophil emigration in rabbits, observed that after injection of LPS, the maximal rate of emigration was consistently 30 min later than with IL-1 or TNF exposure, suggesting that neutrophil emigration in response to LPS was cytokine mediated. However, Lachman (1983) reported that monocytes but not PMNs release IL-1 in response to LPS; therefore, part of the response to LPS in this unfractionated blood system could be due to interaction between monocytes and neutrophils via the release of cytokines.

Previous investigators have used flow cytometry to assess neutrophil functions in normal individuals and in patients with acute infection by both Gram positive and Gram negative organisms. Increased oxidative burst has been reported in patients with documented bacterial infections (Bass 1984, Bassoe 1984, Smedly 1986). However, Bassoe (1984) reported decreased phagocytosis related fluorescence in patients with documented bacterial infections, while Nagel (1986) demonstrated that PMNL from elderly patients have a larger population of nonphagocytic cells. The apparent decrease was suggested by Bassoe (1984) to be due to reduction of phagosomal pH and the sensitivity of FITC to pH.

Simms (1989) examined PMNL and monocytes from 20 patients with acute bacterial infection for phagocytic function. Markedly enhanced phagocytosis as measured by the ingestion of (E) IgG and IgG / C3b - coated erythrocytes was seen. The kinetics of phagocytosis by patient's monocytes was faster. The PMNL and monocytes of these patients are either upregulated with respect to phagocytic function or are less susceptible to downregulation than are normal cells. This probably has a beneficial effect on host defences during infection.

Previously LPS at 10 μg/ml has been shown to activate cells in a unfractionated blood assay system (Trinkle 1987) and to produce an increase in oxidative burst and phagocytosis. We have shown that with the addition of 1 ng/ml LPS or, in some individuals, concentrations as low as 100 pg/ml, we could detect increased activity in both neutrophil functions when compared to a paired co- incubated LPS free control. These concentrations of LPS mimic levels of LPS detected in patients with bacterial sepsis (Levin 1970).

The cationic antibiotic polymyxin B (Poly B) is well known for its ability to block the stimulatory effect of LPS by binding the lipid A portion of the LPS molecule. The mechanisms are still not fully understood. The production of tumor necrosis factor (TNF-α) by isolated rat macrophages in response to LPS and macrophage activating factor was blocked by Poly B (Stokes 1989). Perhaps due to TNF mediating a lot of effects of LPS, this inhibition provides a mechanism to explain the protection afforded by Poly B against LPS induced toxicity. Morrison (1976) found a stoichiometric interaction between LPS and Polymyxin B in a serum-free buffer system, suggesting the binding of one molecule of Poly B to one monomer unit of LPS. Lachman (1983) demonstrated that human serum interfered with Poly B inhibition of LPS activation if LPS was incubated with Poly B prior to addition of serum. We determined that the ratio of Poly B to LPS needed to be 1000:1 or greater for consistent abrogation of the LPS effect in unfractionated blood. We could demonstrate this inhibition by adding Poly B to unfractionated blood immediately prior to addition of LPS.

The use of heparin as an anticoagulant was investigated and found to be less desirable for measuring oxidative burst by DCF fluorescence than ACD. This appears to be due to insufficient dye loading in the presence of heparin. This may be related to the pH of heparinized blood which is higher than its ACD counterpart (pH 7.9 and 7.2, respectively in one individual). The anticoagulant EDTA inhibited phagocytosis and oxidative burst (70 % and 87%, respectively) at concentrations present in CBC tubes. Concentrations less than 20 ug/ml had little effect on unfractionated blood phagocytosis.

Another possible application for this method is to detect neutrophil activation in patients with infection to rapidly confirm suspected endotoxaemia. In addition, it would be of interest to investigate whether "detoxified" LPS derivatives, monophosphoryl lipid A demonstrate priming effects on neutrophils similar to that seen with LPS.

Lipopolysaccharide mutants have been produced and been used to treat patients with Gram-negative bacteraemia. It appears from a study by Ziegler (1982) that the intravenous administration of antisera prepared with a mutant of Escherichia coli which produces lipopolysaccharide with defective side chains, substantially reduces the deaths as a result from Gram negative bacteraemia. In a randomized, double blind, multicenter trial comparing the efficacy of human IgG antibody to Escherichia coli (J5-IVIG) with that of a standard IgG preparation (IVIG), J5-IVIG was not superior to IVIG in reducing mortality or in reversing Gram-negative septic shock (Callandra 1988)

In summary, we have shown that this unfractionated blood flow cytometric method of phagocytosis and oxidative burst determination is an appropriate vehicle to study the relationship between immunomodulatory (e.g. LPS) or chemotherapeutic (Poly B) agents and their effects on these neutrophil functions. This can be carried out on single-laser flow cytometer. It is a rapid, labor-efficient and relatively inexpensive method which avoids the likelihood of inadvertent cellular activation by eliminating the necessity of PMN separation and its adverse mechanical effects. Our flow cytometric assay method for assessment of the direct effects of antibiotics on leukocytes gave comparable results to other tedious and laborious methods described in the literature. The advantages of the flow cytometry method are that it approaches physiological conditions, utilizes unfractionated blood, gives rapid results and is less labor intensive.We have found that the sensitivity of this method depends on appropriate venipuncture and anticoagulants, and most importantly, pyrogenfree agents.

FIGURE LEGENDS

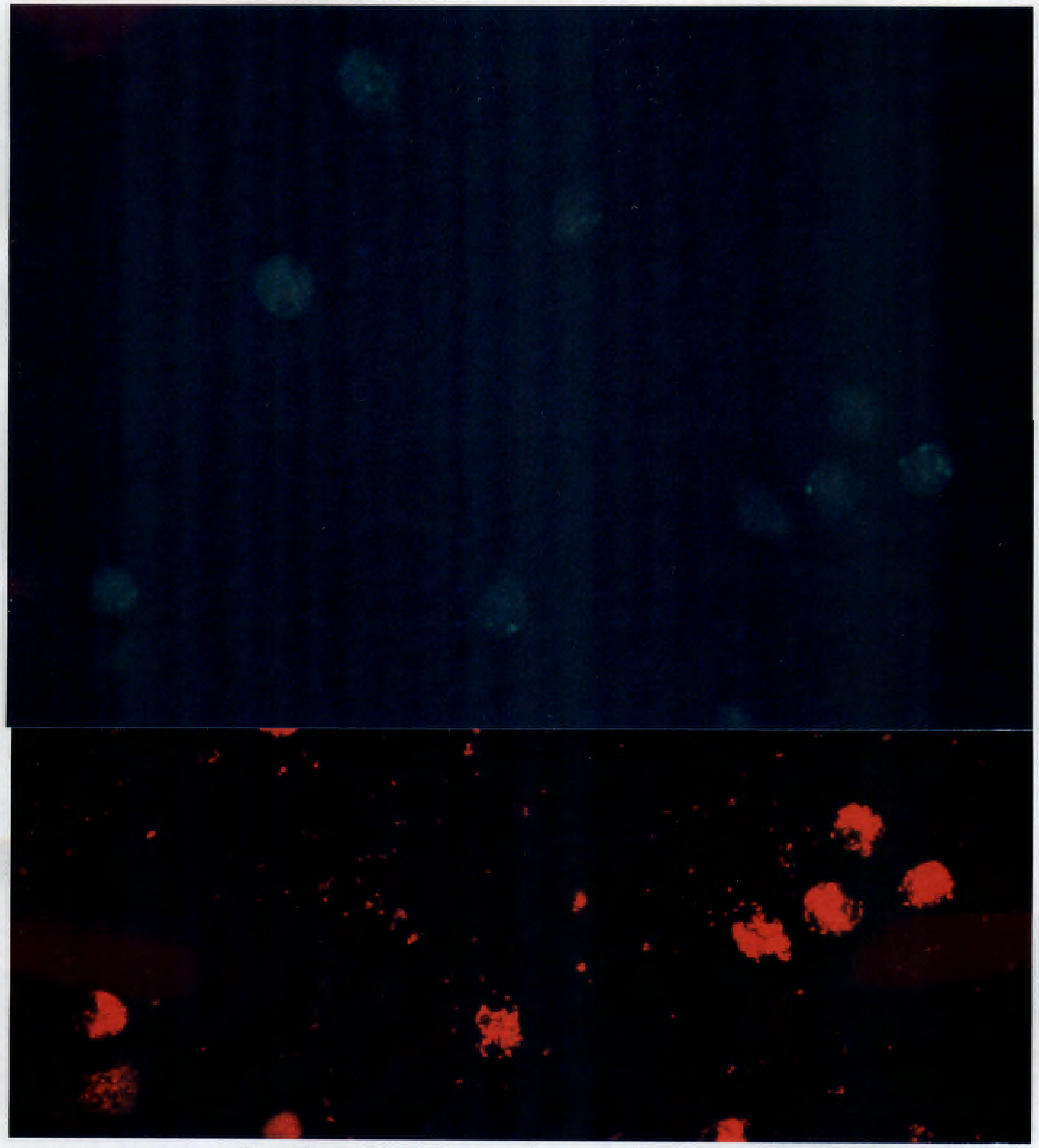


Fig. 1: Fluorescence microscope micrograph of DCF-related fluorescence 10 minutes after addition of DCF-DA to neutrophils (in green); and demonstration of ingested propidium iodide labelled Staph. aureus shown in red.

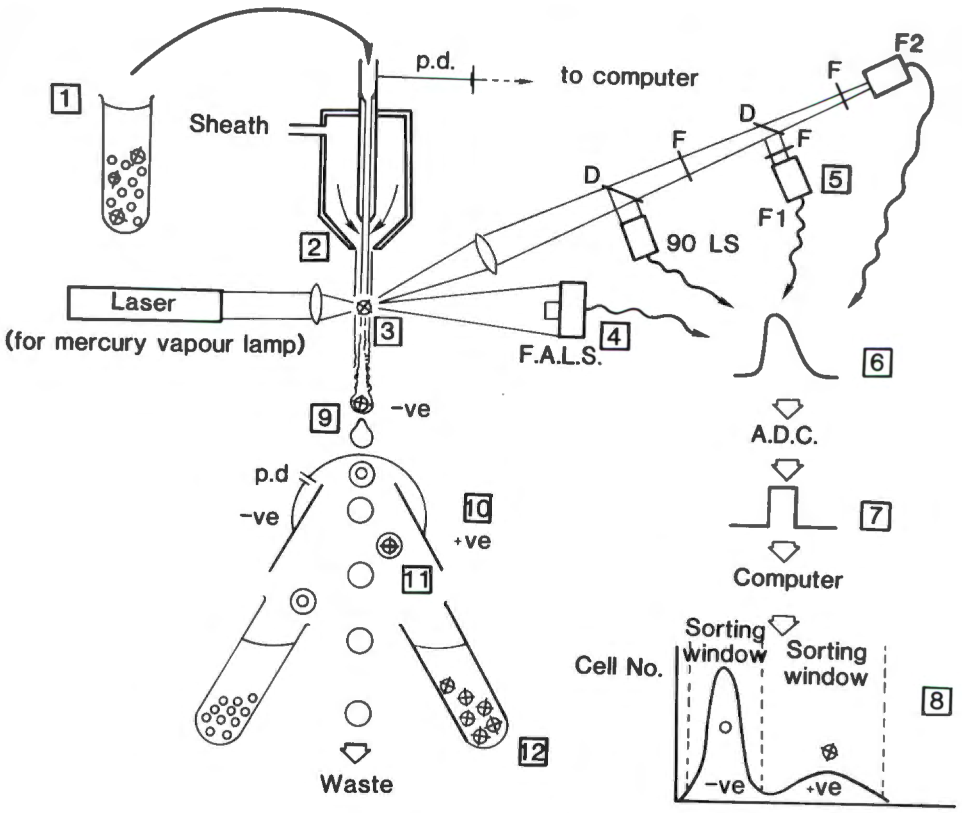


Fig. 2: A diagrammatic representation of a flow cytometer. (compiled from various sources)

1. Represents the sample to be sorted.
2. Represents the narrow bore tube through which stained cells move in single file in the centre of a stream.
3. Detection chamber
4. Forward angle light scatter detector (FALS) with obscuration bar to protect FALS detector from exposure to the direct laser beam.
5. Fluorescence detectors: Typical instruments have three or four of these, one of which is used to measure light scattered perpendicular to the laser beam by the cells.
6. Signal processors.
7. Integral computer which digitizes the signals, stores and displays them.
8. Computer screen display of sorting window.

9-12. Cell sorting facilities.

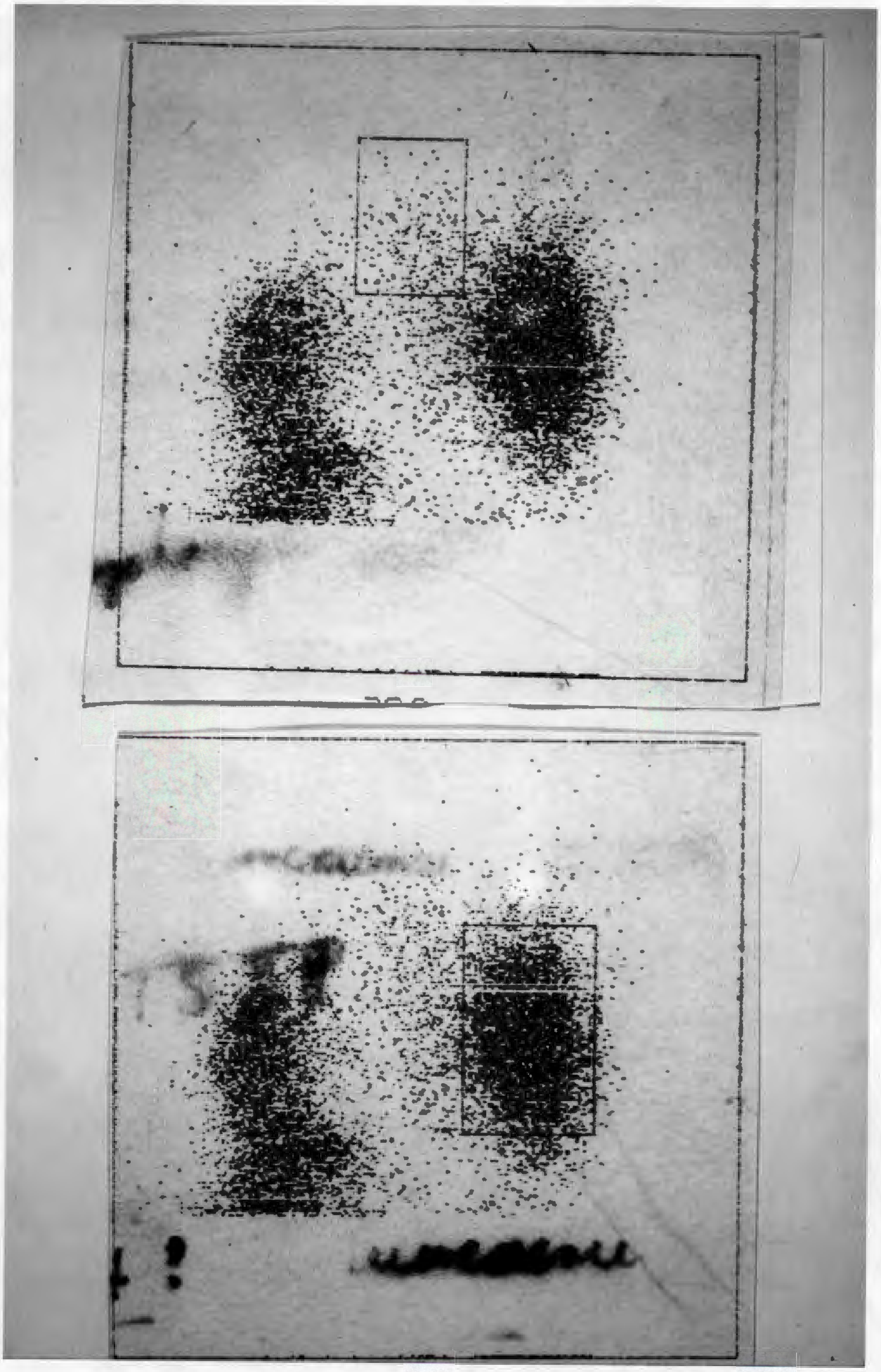


Fig. 3: Flow cytometric scattergram as seen on the computer screen to demonstrate gating of the monocyte region in the top figure and gating of polymorphonuclear leukocytes in the bottom figure.

Fig. 4a.

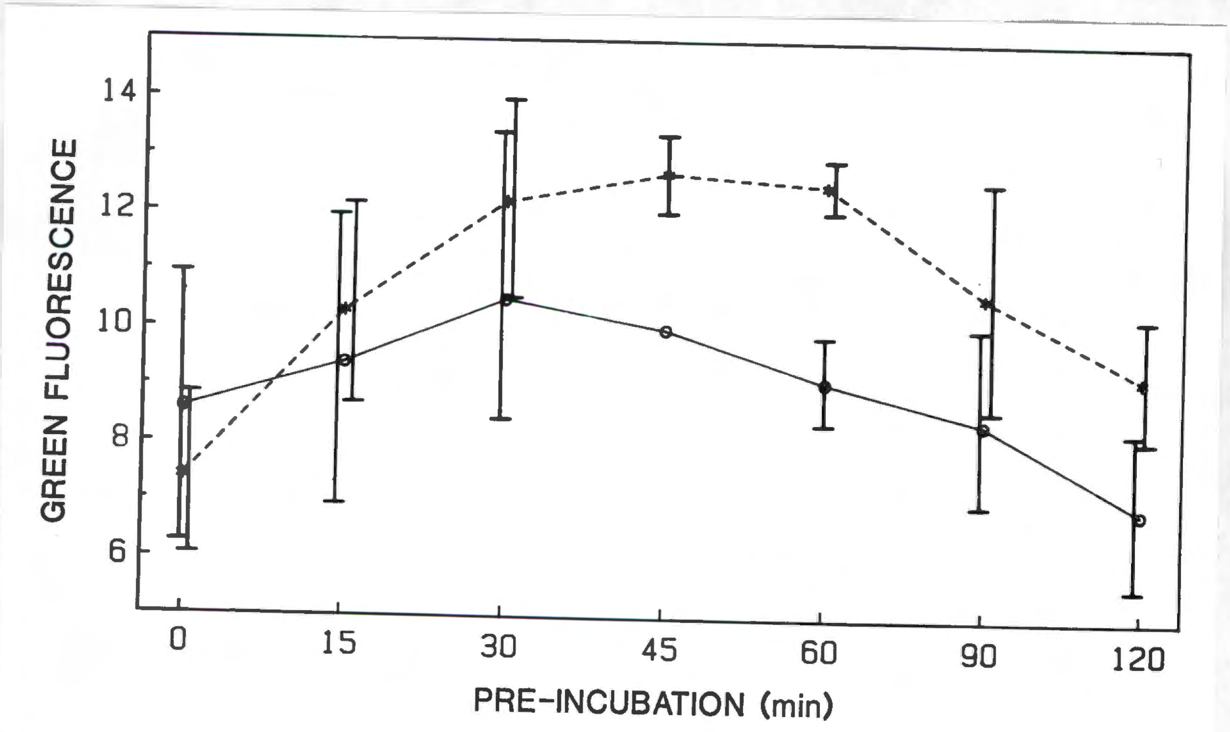

GREEN FLUORESCENCE
14
12
10
8
6
0
15
30
45
60
90
120
PRE-INCUBATION (min)

Fig. 4b.

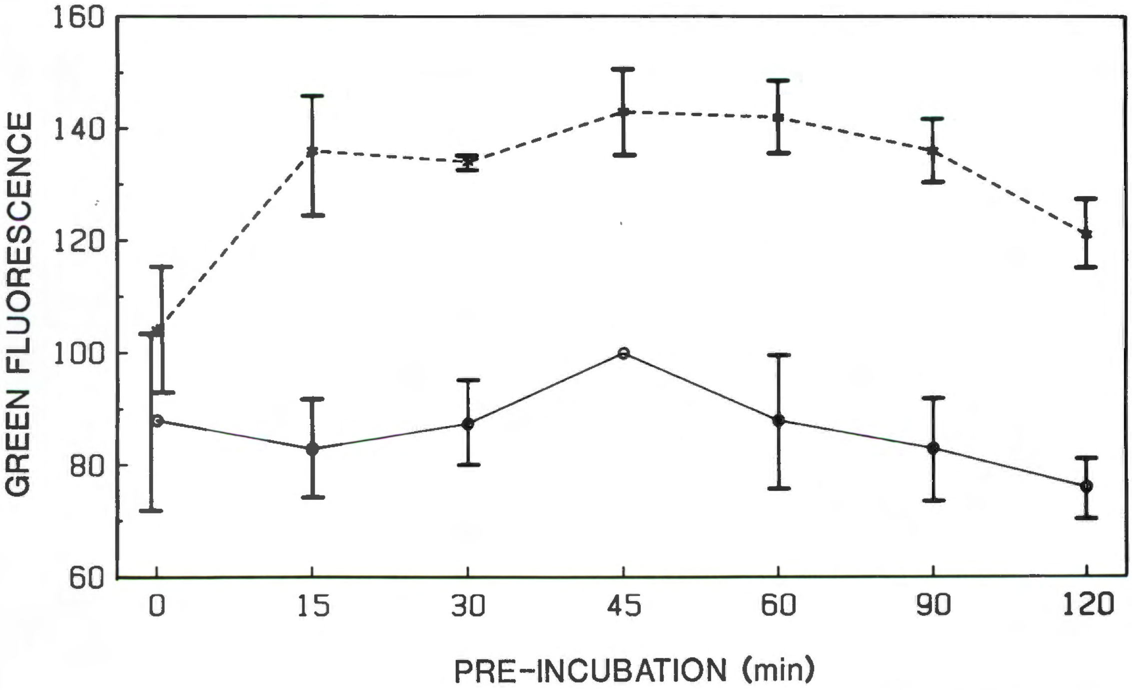

160
140
120
100
80
60
GREEN FLUORESCENCE
0
15
30
45
60
90
120
PRE-INCUBATION (min)

Fig. 4c.

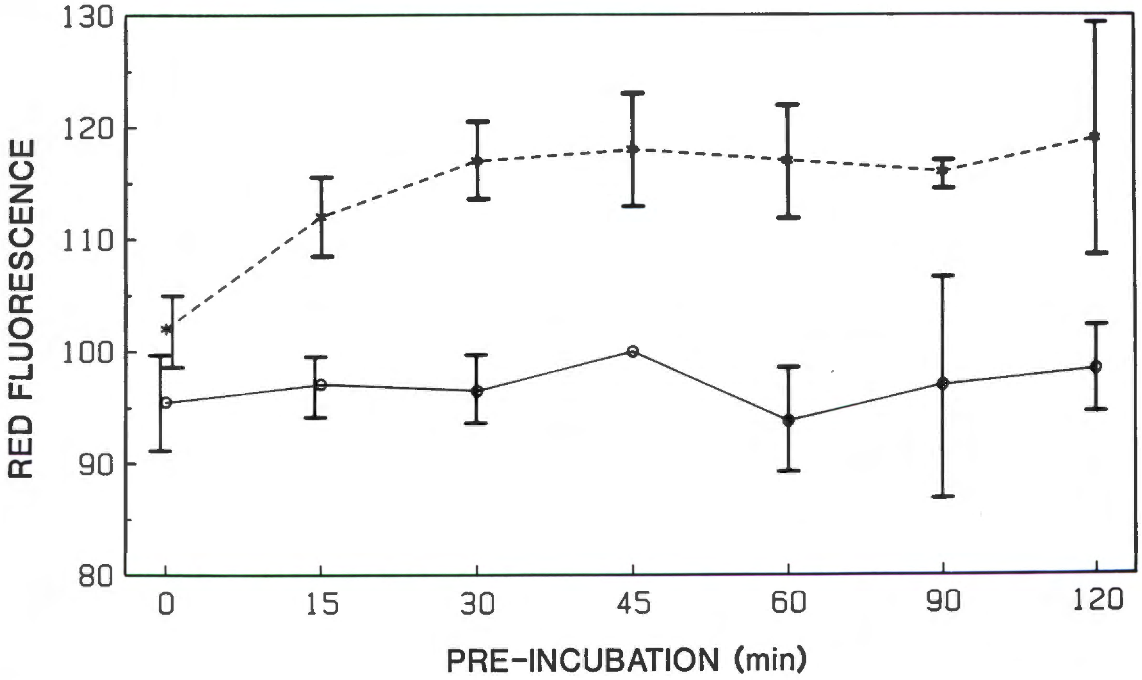


Fig. 4: Time study of LPS effects on phagocytosis and oxidative burst by PMNs. Unfractionated blood from four individuals was pre-incubated with/without LPS from 0-120 minutes (x-axis) prior to addition of 500 μM DCFH-DA.
4a. DCF-related green fluorescence (Y-axis) of samples taken prior to the addition of bacteria (t_0 DCF): (t_0 DCF:_____ ; t_0 DCF + LPS:-------).
4b. DCF-related green fluorescence (Y-axis) of samples taken 10 minutes after the addition of bacteria (t_{10} DCF):
(t_{10} DCF : _____ ; t_{10} DCF + LPS:----).
4c. Red fluorescence related to phagocytosed PI labeled <u>S. aureus</u> (Y-axis) (t_{10} PHAG): (t_{10} PHAG : _____ ; t_{10} PHAG + LPS:-------).

Fig. 5

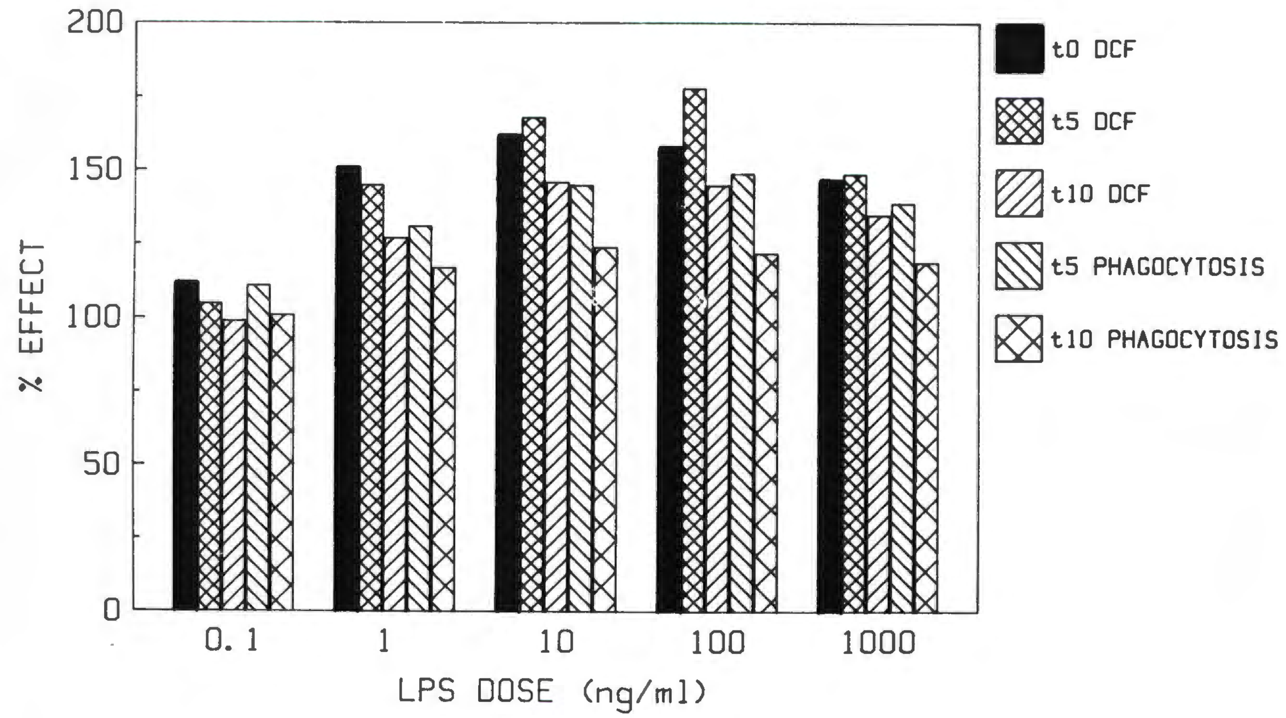


Fig. 5: Dose effects of different LPS concentrations varying from 0,1 to 1000 ng/ml on oxidative burst (DCF) and phagocytosis of PMNs in unfractionated blood measured at different time intervals (0, 5, 10 minutes) after initiation of phagocytosis by addition of propidium-iodide labeled *Staph. aureus*. Results are expressed as the ratio of the measured effect of an LPS-free control to the measured effect of the LPS treated sample in percentage (n = 8).

Fig. 6

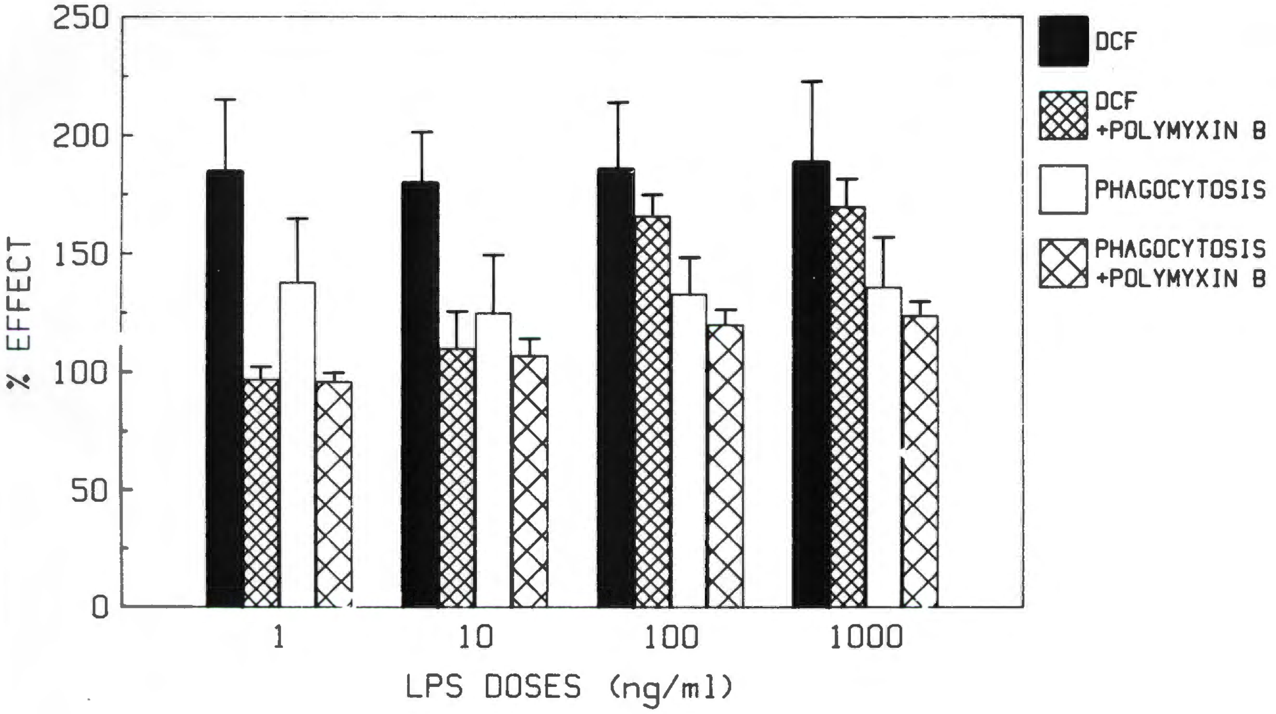


Fig. 6: Effects of LPS in unfractionated blood with/without Polymyxin B (10 μg/ml) on oxidative burst (DCF) and phagocytosis by PMNs. Results are expressed relative to an LPS-and Polymyxin-free control.

Fig. 7

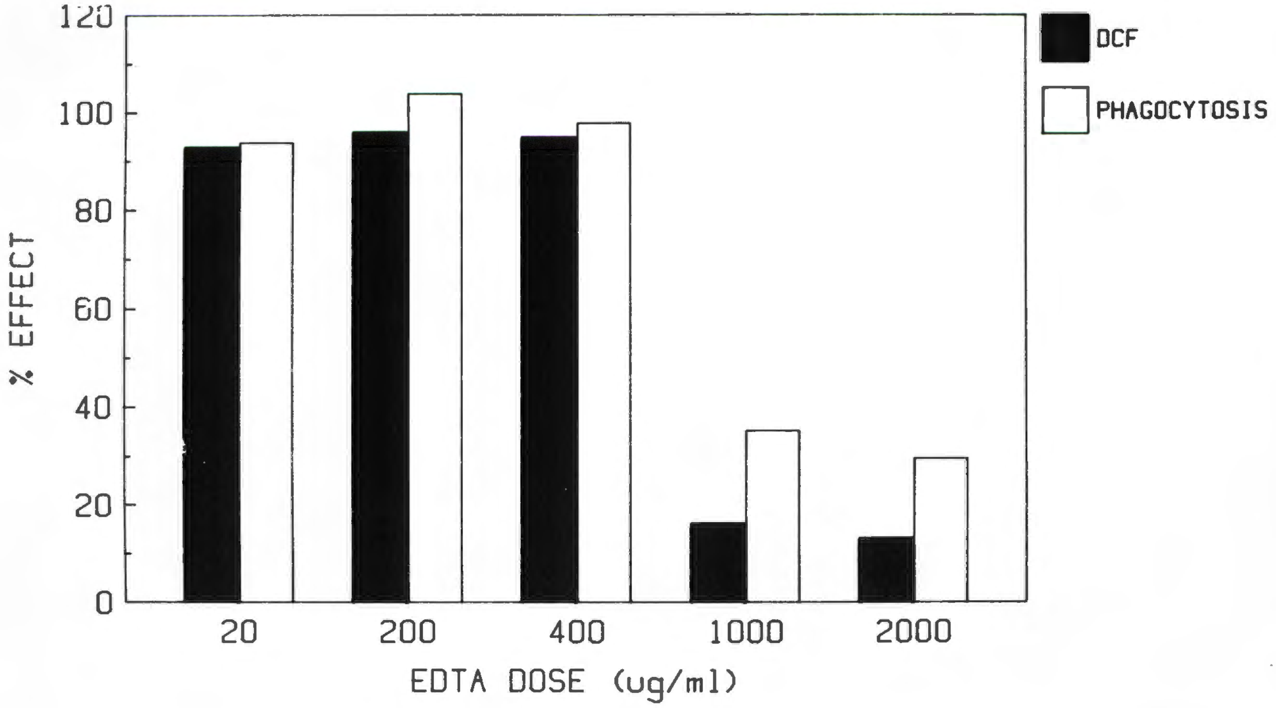
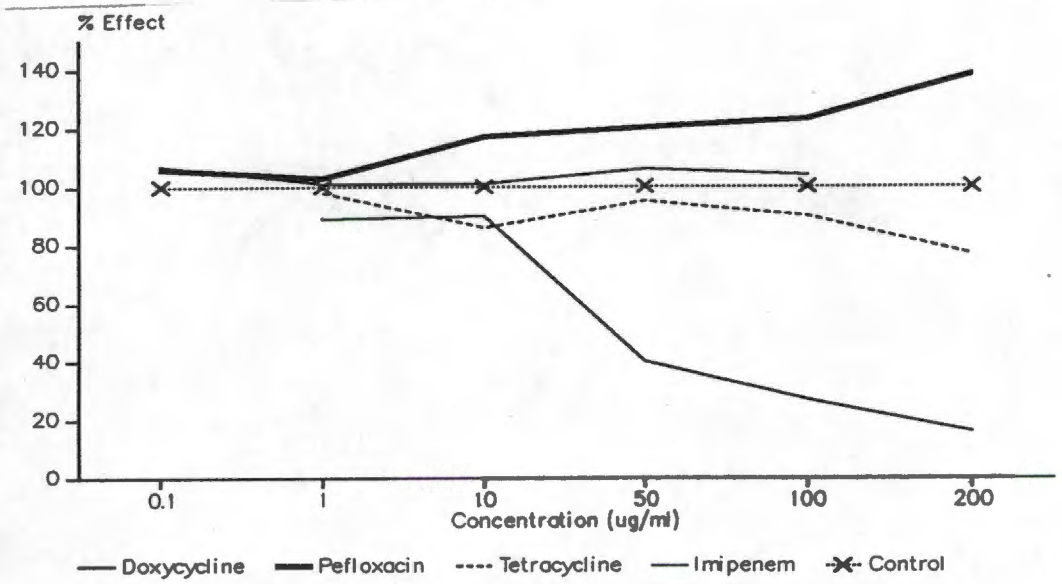


Fig. 7: Dose effects of EDTA (20-2000 μg/ml) on oxidative burst and phagocytosis by PMNs. Results are expressed as a ratio of the measured effect of an EDTA-free unfractionated blood sample relative to a EDTA-treated control

Oxidative Burst



Phagocytosis

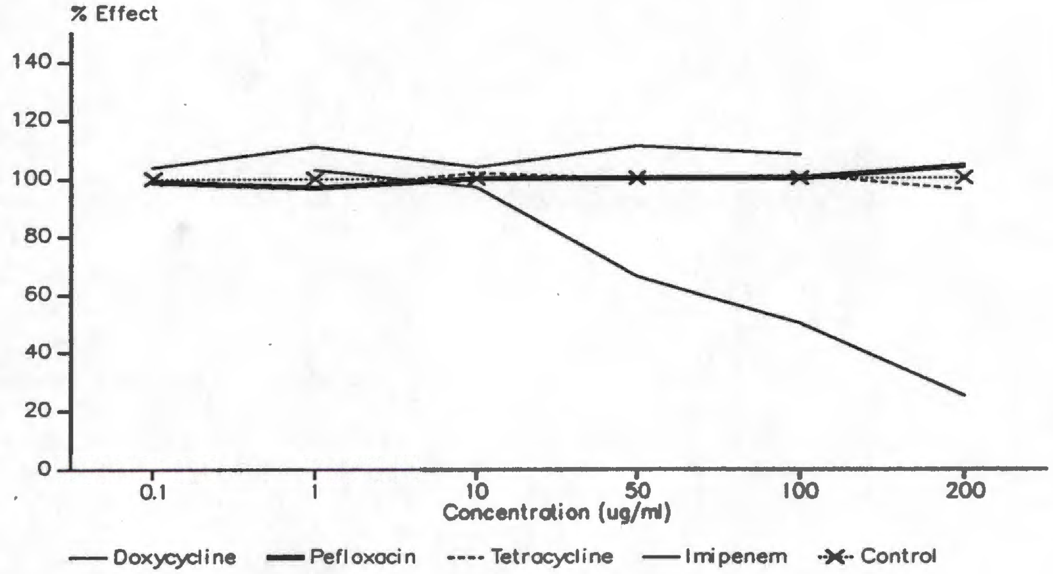


Fig. 8: Dose dependent modulation of oxidative burst and phagocytosis by doxycycline, pefloxacin, tetracycline and imipenem. Results for oxidative burst expressed as the means of the DCF related green fluorescence of the antibiotic exposed cells relative to the means of the DCF related green fluorescence of antibiotic free control cells.Results for phagocytosis expressed as the means of the red fluorescence (RF) of the antibiotic exposed cells relative to the means of RF of the antibiotic free control.

TABLE 1 :EFFECT OF ANTIBIOTICS ON OXIDATIVE BURST BY PMNs AFTER 60 MINUTES INCUBATION AT 37 DEGR C

concentration (µg/ml)

ANTIBIOTICS	200			100			50			20			10			1			0.1		
	a n	b x	c range	n	x		n	x		n	x		n	x		n	x		n	x	
CEFTRIAXONE	7	109	97-126	8	103	89-118	5	99	90-103	-	---	-------	5	98	85-112	5	98	94-106	-	---	-------
CIPROFLOXACIN5	-	---	-------	7	110	92-123	5	100	92-112	-	-98	92-105	5	87	72-93	4	91	89-91	-	---	-------
CLINDAMYCIN	-	---	-------	-	---	-------	-	---	-------	7	98	73-122	8	97	81-109	7	100	90-118	-	---	-------
DOXYCYCLINE	4	16	10-22	5	27	20-40	5	40	21-60	-	---	-------	5	90	86-114	3	89	77-126	-	---	-------
ENOXACIN	-	---	-------	-	---	-------	-	---	-------	5	92	66-105	5	91	82-110	6	104	101-131	5	109	88-147
IMIPENEM	-	---	-------	4	104	93-115	4	106	84-139	-	---	-------	6	101	82-125	5	101	91-128	3	107	99-123
NORFLOXACIN	6	86	77-110	8	94	76-126	5	89	82-106	-	---	-------	4	98	93-104	5	99	91-111	-	---	-------
PEFLOXACIN	6	185	95-420	6	150	101-286	-	---	-------	-	---	-------	6	130	104-193	6	106	86-117	6	115	104-162
TEICOPLANIN	4	103	100-106	5	91	80-100	5	94	86-101	-	---	-------	5	100	86-110	4	100	92-109	-	---	-------
TETRACYCLINE	6	77	60-86	8	90	81-107	7	95	77-111	-	---	-------	7	86	75-100	4	98	91-104	-	---	-------
VANCOMYCIN	4	102	98-107	4	101	95-104	4	106	90-115	-	---	-------	4	109	97-126	4	113	98-127	-	---	-------

a. Number of determinations.

b. Mean of percent fluorescence of antibiotic exposed cells relative to percent fluorescence of antibiotic free control cells.

c. Range of percent fluorescence of antibiotic exposed cells relative to percent fluorescence of antibiotic free control cells.

TABLE 2:EFFECT OF ANTIBIOTICS ON PHAGOCYTOSIS BY PMNs AFTER 60 MINUTES INCUBATION AT 37 DEGR C

Concentration (μg/ml)

ANTIBIOTICS	200			100			50			20			10			1			0.1		
	n[a]	x[b]	range[c]	n	x		n	x		n	x		n	x		n	x		n	x	
CEFTRIAXONE	7	109	97-134	8	105	87-124	5	105	98-115	-	---	-------	5	106	92-129	4	109	102-117	-	---	-------
CIPROFLOXACIN	-	---	-------	7	97	82-111	5	102	95-104	4	96	89-102	5	96	84-106	4	95	88-104	-	---	-------
CLINDAMYCIN	-	---	-------	-	---	-------	-	---	-------	7	97	86-116	8	94	85-113	7	101	95-116	-	---	-------
DOXYCYCLINE	4	25	14-32	6	50	41-65	5	66	57-82	-	---	-------	4	97	86-108	2	103	95-110	-	---	-------
ENOXACIN	-	---	-------	-	---	-------	-	---	-------	5	99	84-110	5	106	94-120	6	105	94-115	5	110	94-139
IMIPENEM	-	---	-------	4	108	96-119	4	111	95-138	-	---	-------	6	104	93-129	6	111	95-131	3	104	100-112
NORFLOXACIN	7	101	84-114	9	100	91-113	6	98	92-105	-	---	-------	5	101	96-112	6	101	99-105	-	---	-------
PEFLOXACIN	6	103	92-113	6	100	90-112	-	---	-------	-	---	-------	6	100	92-110	6	97	92-104	6	97	84-104
TEICOPLANIN	4	101	91-118	5	98	89-108	5	96	93-107	-	---	-------	5	100	91-119	4	99	88-109	-	---	-------
TETRACYCLINE	6	96	88-115	8	101	93-114	7	100	86-132	-	---	-------	7	102	89-116	4	97	95-101	-	---	-------
VANCOMYCIN	4	99	94-106	4	102	97-110	4	101	95-104	-	---	-------	4	106	99-114	4	105	99-114	-	---	-------

a.Number of determinations.

b.Mean of percent fluorescence of antibiotic exposed cells relative to percent fluorescence of antibiotic free control cells.

c.Range of percent fluorescence of antibiotic exposed cells relative to percent fluorescence of antibiotic free cells.

LITERATURE CITED:

Athlin, L., L. Domellöf, and B. Norberg. (1984). Adherence and phagocytosis of yeast cells by blood monocytes effects in vitro of therapeutic doxycycline concentration. J.Immunol. Scand. Sect. C 92:227-230.

Babior, B.M. (1982). The Role of Active Oxygen in Microbial Killing by Phagocytes. In: Pathology of Oxygen, Author, A.P. (ed.), Academic Press, Inc., New York, 45-56.

Bass, D.A., J.W. Parce,L.R. Dechatelet,P. Szejda, M.S. Seeds, M.Thomas. (1983). Flow cytometric studies of oxidative product formation by neutrophils: A graded response to membrane stimulation. J Immunol 130:1910-1917.

Bass, D.A., P. Olbrantz, P. Szejda, M.C. Seeds, and C. E. McCall. (1984). Subpopulations of neutrophils with increased oxidative product formation in blood of patients with infection. J. Immunol. 136:860-866.

Bassoe, C.F. (1984). Flow cytometric studies on phagocyte function in bacterial infections. Acta Path. Microbiol. Immunol. Scand. Sect. C 92:167-171.

Bassoe, C. F., O. D. Laerum, C. O. Solberg, and B. Haneberg. (1983). Phagocytosis of bacteria by human leukocytes measured by flow cytometry (41722). Proc. Soc. Exper. Biol. Med. 174:182-186.

Bassoe, CF, R.Bjerknes. (1985). Phagocytosis by human leukocytes, phagosomal pH and degradation of seven species of bacteria measured by flow cytometry. J Med Microbiol 19:115-125.

Bassoe, C.F., and C.O.Solberg. (1984). Phagocytosis of *Staphylococcus aureus* by human leukocytes: Quantitation by a flow cytometric and a microbiological method. Acta Pathol. Microbiol. Immuno. Scand. Sect. C 92:43-50.

Belsheim, J., C. Blomqvist, J. Löfberg, and H. Gnarpe. (1983). Tetracycline influence on leukocyte functions. Acta Oto-Rhino-laryngologica Belgica 37:635-648.

Bjerknes, R., C.F. Bassoe. (1984). Phagocyte C3-mediated attachment and internalization: Flow cytometric studies using a fluorescence quenching technique. Blut 49:315-323.

Boogaerts, M.A., S. Malbrain, W. Scheers, R.L. Verwilghen. (1986). Effects of quinolones on granulocyte function in vitro. Infection 14 (Suppl. 4):258-262.

Calandra, T., M.P. Glauser, J.Schellekens. (1988). Treatment of Gram-negative Septic Shock with Human IgG Antibody to *Escherichia coli* J5: A Prospective, Double-blind, Randomized Trial. J. Infectious Diseases. Vol 158:No 2,312-319.

Cybulsky, M.I., D.J. McComb, H.Z. Movat. (1988). Neutrophil leukocyte emigration induced by endotoxin.Mediator roles of interleukin 1 and tumor necrosis factor Á. J Immunol 140:3144-3149.

Dahinden, C., C. Galanos, J. Fehr. (1983). Granulocyte activation by endotoxin. J Immunol 130:857-862.

Daschner, F.D. (1985). Antibiotics and host defence with special reference to phagocytosis by human polymorphonuclear leukocytes. J. Antimicrobial Chemotherapy 16: 135-141.

Davis, D.B., R. Dulbecco, H.N. Eisen, H.S. Ginsberg. (1980). Microbiology, 3rd edition, Harper and Row, 82-85.

Desnottes, J.F., F. Jacotot, J. Bruel, M.T. Bassoullet, G. Niel. (1986). Effects of pefloxacin on phagocytosis function of rat macrophages and polymorphonuclear leucocytes. J. Antimicrobial Chemother. 17 (suppl. B):53-57.

Duncker, D., and U. Ullmann. (1986). Influence of various antimicrobial agents on the chemiluminescence of phagocytosing human granulocytes. Chemotherapy 32:18-24.

Dunn, P.A., and H.W. Tyrer. (1981). Quantitation of neutrophil phagocytosis, using fluorescent latex beads. J. Lab. Clin. Med. 98: 374-381.

Fearon, D.T., and L.A. Collins. (1983). Increased expression of C3b receptors on polymorphonuclear leukocytes induced by chemotactic factors and by purification procedures. J. Immunol. 130 No 1:370-375.

Fietta, A., C. Bersani, V. DeRose, F.M. Grassi, G.G. Gialdroni. (1986). The effect of teicoplanin on leukocytic activity and intraleukocytic micro-organisms. J. Hosp. Infect. 7 (A):57-63.

Forsgren, A. (1984). Antimicrobial agents and host defence. Scand. J. Infect. Dis. Suppl. 43:24-33.

Forsgren, A., and P.I. Bergkvist. (1985). Effect of ciprofloxacin on phagocytosis. Europ. J. Clin. Microbiol. 4 (6):575-578.

Forsgren, A., D. Schmeling, and P.G. Quie. (1974). Effect of tetracycline on the phagocytic function of human leukocytes. J. Infect. Dis. 130: 412-415.

Geisen, H.P., M. Schipplick, J. Schömig-Breckner, M. Betzler. (1986). Granulozytenfunktion unter dem Einfluss von Antibiotika. Beitr. Infusionstherapie klin. Ernähr 15: 277-284.

Gnarpe, H., and D. Leslie. (1974). Tetracyclines and host defence mechanisms. Doxycycline interference with phagocytosis of *Escherichia coli.* Microbios 10A: 127-138.

Grec, V., P.C. Frei. (1984). Effect of amoxycillin and doxycycline on function of human granulocytes tested in vitro and on chemotaxis of granulocytes from rabbits given the two antibiotics. Inflammation 8 No.4:417-427.

Guthrie, L.A., L.C. Mcphail, P.M. Henson, and R.B. Johnston Jr. (1984). Priming of neutrophils for enhanced release of oxygen metabolites by bacterial lipopolysaccharide. J. Exp. Med. 160:1656-1671.

Haslett, C., L.A. Guthrie, M.M. Kopaniak, R.B. Johnston Jr., P.M. Henson. (1985). Modulation of multiple neutrophil functions by preparative methods or trace concentrations of bacterial lipopolysaccharide.Am.J. Pathol. 119:101-110.

Hed, J., G. Hallden, S.G.O. Johansson, P. Larsson. (1987). The use of fluorescence quenching in flow cytofluorometry to measure the attachment and ingestion phases in phagocytosis in peripheral blood without prior cell separation. J. Immunol. Meth. 101:119-125.

Keston, A.S., R. Brandt. (1965). The fluorometric analysis of ultramicro quantities of hydrogen peroxide. Anal Biochem 11:1-5.

Lachman, L.B. (1983). Interleukin-1 Release from LPS-stimulated mononuclear phagocytes. In: Beneficial Effects of Endotoxins, A. Nowotny (ed.), Plenum Press, NY, 283-305.

Levin, J., E.Poore, N.P. Zanber, R.S. Oser. (1970). Detection of endotoxin in the blood of patients with sepsis due to Gram-negative bacteria. N Engl J Med 283 No 24:1313-1316.

Melby, K., and T. Midtvedt. (1981). Studies on the phagocytic activity of human and rat polymorphonuclear cells exposed to doxycycline in vivo. Chemotherapy 27:452-458.

Milatovic,D. (1983). Antibiotics and phagocytosis. Eur. J. Clin. Microbiol. 2 No.5: 414-425.

Morrison, D.C., D.M. Jacobs. (1976). Binding of polymyxin B to the lipid A portion of bacterial lipopolysaccharides. Immunochemistry 13:813-818.

Nagel, J.E., K.Han, P.J. Coon, W.H. Adler, and B.S. Bender. (1986). Age differences in phagocytosis by polymorphonuclear leukocytes measured by flow cytometry. J. Leukocyte Biol. 39:399-407.

Natanson C., R.L. Danner, R.J. Elin, J.M. Hosseini, K.W. Peart, S.M. Banks, T.J. MacVittie, R. I. Walker, J.E. Parrillo. (1989). Role of Endotoxaemia in Cardiovascular Dysfunction and Mortality. J. of Clinical Invest. 243 : 243-251.

Quirke, P., and J.E.D. Dyson. (1986). Flow cytometry: Methodology and applications in pathology.J. Pathol. 149: 70-87.

Ribi, E. (1984). Beneficial Modification of the Endotoxin molecule. J Biological Response Modifiers 3:1-9.

Seneca, H., and P. Peer. (1966). Effect of antibacterials, antibiotics, enzymes, and steroids on phagocytosis. J. Am. Geriat. Soc. XIV No. 3: 187-199.

Shenep J.L., Mogan K.A. (1984). Kinetics of Endotoxin Release During Antibiotic Therapy For Experimental Gram-Negative Bacterial Sepsis. J. Inf. Dis. 150:380-388.

Siegel, J.P., and J.S. Remington. (1982). Effect of antimicrobial agents on chemiluminescence of human polymorphonuclear leucocytes in response to phagocytosis. J. Antimicrob. Chemother. 10: 505-515.

Simms H.H., M.M.Frank, T.C. Quinn, S. Holland, T.A. Gaither. (1989). Studies on phagocytosis in patients with acute bacterial infections. J.Clin.Invest. Vol. 8: 252-260.

Smedly L.A., M.G. Tonnesen, R.A. Sandhaus, C. Haslett, L.A. Guthrie, R.B. Johnston Jr, P.M. Henson, G.S.Worthen. (1986). Neutrophil-mediated injury to endothelial cells. J Clin Invest 77:1233-1243.

Stokes D.C., J.L.Shenep, M.Fishman, W.K.Hildner, G.K.Bysani, K.Rufus. (1989). Polymyxin B prevents Lipopolysaccharide-Induced Release of Tumor Necrosis Factor-α from Alveolar Macrophages. Vol.160.No.1:52-57.

Suffredini A.F., R.E. Fromm, M.M. Parker, M. Brenner, J.A.Kovacs, R.A. Wesley, J.E.Parrillo. (1989). The cardiovascular response of normal humans to the administration of endotoxin. N Engl J Med Vol.321 No.5: 280-287.

Szejda, P., J.W. Parce, M.S. Seeds, and D.A. Bass. (1984). Flow cytometric quantitation of oxidative product formation by polymorphonuclear leudocytes during phagocytosis. J. Immunol. 133:3303-3307.

Trinkle, L.S., S.R. Wellhausen, and K.R. McLeish. (1987). A Simultaneous Flow Cytometric measurement of neutrophil phagocytosis and oxidative burst in whole blood. Diag. Clin. Immunol. 5:62-68.

Weiss S.J. (1989). Tissue destruction by neutrophils.N Engl J Med Vol.320 No. 6: 365-376.

Wilson, M.E., D.P. Jones, P. Munkenbeck, D.C.Morrison. (1982). Serum-dependent and independent Effects of bacterial Lipopolysaccharides on human Neutrophil Oxidative Capacity In Vitro. J Reticuloendothelial Soc 31:43-57.

Wilson, R.M., A.M. Galvin, R.A. Robins, W.G. Reeves. (1985).Flow cytometric Method for the Measurement of Phagocytosis by Polymorphonuclear Leucocytes. Vol.76: 247-253.

Wood, D.D., P.M. Cameron. (1978). The relationship between bacterial endotoxin and human B cell activating factor. J Immunol 121: 53-60.

Wolff S.M. (1982). The treatment of Gram-Negative bacteremia and Shock. Editorial. N Engl J Med Vol. 307 No. 20: 1267-1268.

Ziegler E.J.. (1988). Protective Antibody to Endotoxin Core: The Emperor's New Clothes.J.of Infectious Diseases. Vol.158: 286- 290.

Ziegler E., J.A.McCutchan, J.Fierer, M.P.Glauser, J.C. Sadoff, H.Douglas, A.I.Braude. (1982). Treatment of Gram-negative bacteremia with human antiserum to a mutant Escherichia coli. N Engl J Med Vol.307 No.20: 1225-1230.