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ABNORMAL RESPONSES OF GRANULOCYTES IN CHRONIC GRANULOMATOUS DISEASE

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Stimulation of normal granulocytes with chemotactic factor, phorbol myristate acetate, concanavalin A, and calcium ionophore results in rapid depolarization which precedes the 'respiratory burst'. Treatment of granulocytes in chronic granulomatous disease with these stimulants fails to generate chemiluminescence. This defect is associated with an absence of transmembrane potential shifts in response to treatment with chemotactic factor, phorbol myristate acetate, and concanavalin A while depolarization in response to A23187 is unaffected by this disease state.

Introduction

Chronic granulomatous disease is a disorder characterized by high susceptibility to bacterial infection [1,2]. This disease has been traced to defects in granulocyte function. These cells fail to kill bacteria even though there is no apparent decrease in bacterial ingestion [3]. Evidence indicates that this disease is characterized by an absence of the 'respiratory burst' associated with phagocytosis by granulocytes [4]. Indeed, granulocytes from patients with chronic granulomatous disease fail to produce antibacterial substances, such as, superoxide anion or hydrogen peroxide, in response to particle-exposure [5,6]. In addition, these cells fail to generate chemiluminescence or increase hexose monophosphate shunt activity in response to bacteria [7].

Recent data indicate that stimulation of granulocytes with chemotactic factors, phorbol myristate acetate, or concanavalin A results in a rapid depolarization followed by a prolonged hyperpolarization of the transmembrane potential [8–11]. In contrast, stimulation with A23187, i.e., a calcium ionophore, results in a rapid and prolonged depolarization [9]. Evidence indicates that these shifts in transmembrane potential precede the release of superoxide anion or the generation of chemiluminescence induced by these stimulants [8–11]. Therefore, it has been suggested that depolarization of the membrane potential may act as a signal which triggers the 'respiratory burst'.

The objective of this investigation is to determine if chronic granulomatous disease alters the transmembrane potential response of granulocytes to stimulants. The stimulants tested were *N*-formyl-methionyl-leucyl-phenylalanine, phorbol myristate acetate, concanavalin A, and A23187.

Materials and Methods

Isolation of granulocytes

Human granulocytes were isolated by dextran sedimentation followed by centrifugal elutriation as described previously [12]. A unit of whole blood was drawn into a blood storage bag containing 63 ml CPD anti-coagulant (1.66 g sodium citrate, 1.61 g

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dextrose, 0.20 g citric acid, and 0.14 g sodium diphosphate). The blood was mixed with 150 ml of a modified Dulbecco's medium (140 mM NaCl, 2.7 mM KCl, 1.47 mM KH_2PO_4, 10 mM Na_2HPO_4, with 9 g dextran (M_r = 100 000–200 000), and 0.5 g gelatin (pH 7.4). This suspension was incubated for 1 h at 37°C to allow settling of red blood cells. After this time, the supernatant was collected and centrifuged at 200 × *g* for 5 min at 2°C to pellet the cells. The cells were immediately resuspended in Dulbecco's medium (without dextran or gelatin). Granulocytes were then separated from the other blood cells in this suspension by centrifuged elutriation at 2°C. Elutriation allows separation of cells into populations of different diameters by balancing centrifugal force against a moving stream of buffer (145 mM NaCl, 5 mM KCl, 9.35 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, and 5 mM glucose (pH 7.4). During these experiments the rotor speed, and therefore, the centrifugal force was kept constant at 2000 rev./min while the flow of fluid was controlled using a variable speed pump. Red cells and lymphocytes were removed at a flow rate of 13.0 ml/min. The remaining cells were washed from the elutriator with 150 ml of buffer at a flow rate of 41 ml/min. This suspension was centrifuged at 480 × *g* for 5 min at 2°C and the supernatant discarded. The cells were resuspended in 10 ml of buffer and a second elutriation, identical to the first, was performed. Granulocytes were then collected from the 41 ml/min fraction as described above. Purity of these granulocyte preparations was approx. 95% with a cell viability of greater than 95%.

Measurement of transmembrane potential

The transmembrane potential of human granulocytes was measured at 22°C using a fluorescent probe, Di-S-C_3(5) [9,12]. This probe was a gift from Dr. Alan Waggoner (Department of Chemistry, Amherst College, Amherst, MA). Fluorescnce was measured with a fluorescent spectrophotometer fitted with a magnetic stirrer. Excitation and emission wavelengths were set at 622 and 665 nm, respectively. All samples contained $2.3 \cdot 10^7$- cells suspended in 3 ml of Hepes medium (145 mM NaCl, 5 mM KCl, 10 mM Na-Hepes, 5 mM glucose, and 1 mM $CaCl_2$ (pH 7.4)). Di-S-C_3(5) (final concentration = 0.66 μg/ml) was added to each suspension and allowed to equilibrate at 22°C until the fluorescence level was steady. Stimulants were then added to the cell suspension and the change in fluorescence monitored. Previous studies have shown that this dye is not toxic to human granulocytes and that stimulants do not react directly with fluorescent dye [12,9].

Measurement of chemiluminescence

Generation of chemiluminescence was measured at 37°C as counts per min (cpm) recorded in the tritium channel of a liquid scintillation counter operated in the out-of-coincidence mode [13]. Each sample contained $1 \cdot 10^6$ cells in 5 ml of Hepes medium plus 10^{-8} M luminol. Stimulants were added to the cell suspensions at the onset of the chemiluminescence assay. Evidence indicates that chemiluminescence correlates well with the 'respiratory burst' [14] and that it is an effective method for monitoring chronic granulomatous disease [15].

Case history

The patient is a 14-year-old white male with normal parents and siblings. Chronic granulomatous disease was diagnosed at 2 years of age by the absence of particle-stimulated reduction of nitroblue tetrazolium dye and by the absence of bacterial killing. Diagnosis was later confirmed by failure of stimulated granulocytes to generate chemiluminescence.

The patient's clincal course consisted of many of the commonon manifestations of the disease, chiefly, chronic, recurrent lymphadenitis, subcutaneous abcesses, seborrheic dermatitis, blepharitis, pneumonitis, hepatic and perihepatic abcesses, and osteomyelitis. Over the years, he showed chronic growth failure (height and weight below the 5th percentile), progressive hepatosplenomegaly, chronic mild leukopenia and mildly hypochromic microcytic anemia, elevated sedimentation rate, hypergamma-globulinemia, and elevated alkaline phosphatase. In time, isolated areas of liver calcification and pulmonary infiltrates were demonstrated by X-ray. To date severe infections have required a total of 16 hospitalizations.

The patient was clinically well and received no drug therapy at the time the blood was drawn for this study.

Results

Recent reports indicate that treatment of normal granulocytes with *N*-formyl-methionyl-leucyl-

phenylalanine (a chemotactic factor), phorbol myristate acetate, concanavalin A (a lectin), or A23187 (a calcium ionophore) stimulates superoxide release and the generation of chemiluminescence [8–11]. The data also indicate that shifts in the transmembrane potential precede this 'respiratory burst' by granulocytes [8–11]. Normal shifts in granulocyte transmembrane potential in response to these stimulants are shown in Fig. 1. Note that an increase in the fluorescence of Di-S-C_3(5) indicates depolarization while a decrease in fluorescence indicates hyperpolarization. *N*-Formyl-methionyl-leucyl-phenylalanine treatment of normal granulocytes results in a rapid depolarization which peaks in less than 1 min followed by a prolonged hyperpolarization. In contrast, *N*-formyl-methionyl-leucyl-phenylalanine treatment of granulocytes from a patient with chronic granulomatous disease does not result in a transmembrane potential shift (Fig. 1A). Similar results were also obtained with concanavalin A (Fig. 1B) and phorbol myristate acetate (Fig. 1C). Granulocytes from healthy donors exhibit biphasic shifts in transmembrane potential, yet concanavalin A treatment fails to induced changes in the potential of diseased granulocytes (Fig. 1B) while phorbol myristate causes only very slight depolarization (Fig. 1C). In contrast to these stimulants, A23187, a calcium ionophore, induces a monophasic (depolarization) rather than a

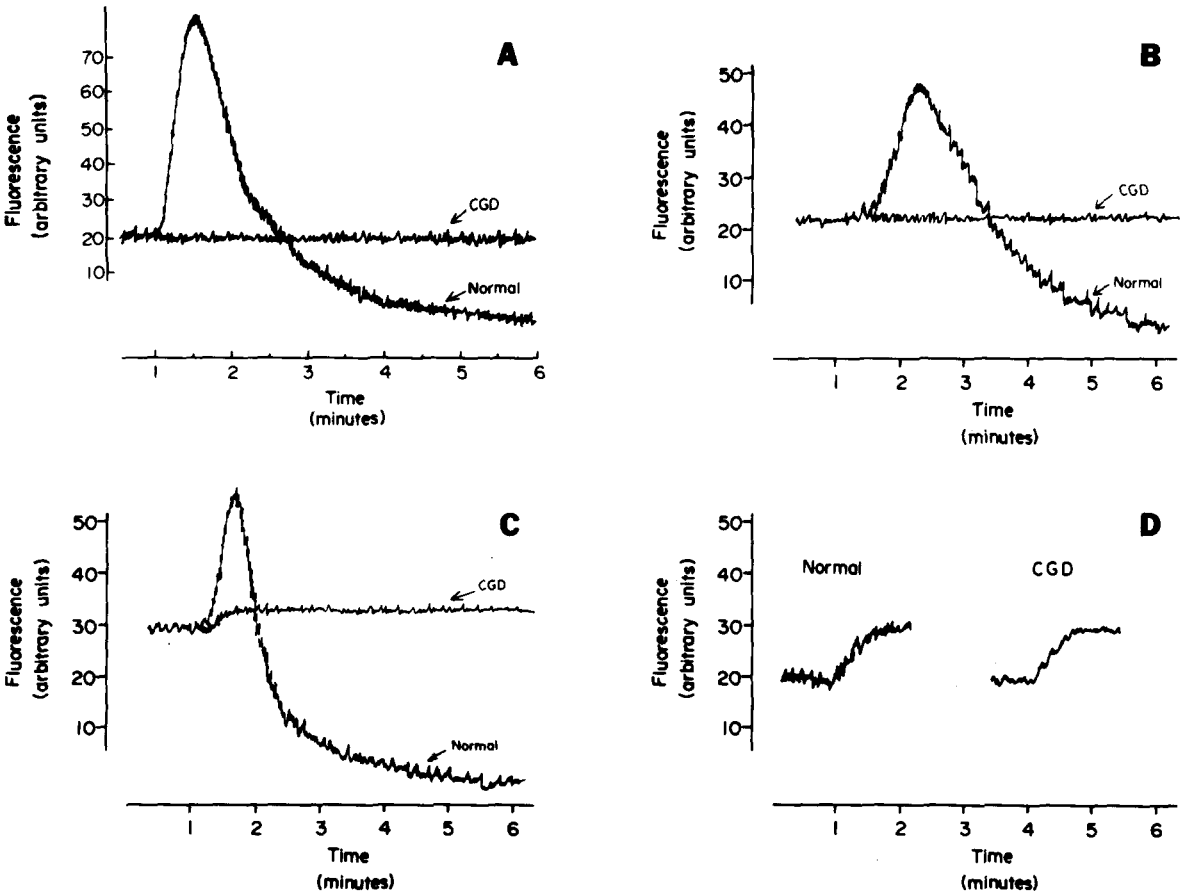

Fig. 1. The effects of stimulants on the transmembrane potential of granulocytes from a healthy donor (normal) and a patient with chronic granulomatous disease (CGD). (A) *N*-Formyl-methionyl-leucyl-phenyalanine ($1.65 \cdot 10^{-8}$ M). (B) Concanavalin A (500 μg/ml). (C) Phorbol myristate acetate (5 μg/ml). (S) A23187 ($5 \cdot 10^{-7}$ M). See Materials and Methods for experimental details.

biphasic shift in the potential of normal granulocytes (Fig. 1D). However, this A23187-induced depolarization is also exhibited by granulocytes from patients with chronic granulomatous disease (Fig. 1D).

Because stimulant-induced depolarization precedes the generation of chemiluminescence and superoxide anion secretion, it has been suggested that membrane depolarization may trigger the release of superoxide anion from granulocytes [8–11]. If this hypothesis is correct, stimulants which fail to induce potential shifts in diseased granulocytes may also fail to induce chemiluminescence. Our results indicate that treatment of diseased granulocytes with $2 \cdot 10^{-5}$ M *N*-formyl-methionyl-leucyl-phenylalanine results in almost no chemiluminescence. It has also been shown that phorbol myristate acetate fails to induce superoxide release from granulocytes in chronic granulomatous disease [16]. In contrast, we find that $1 \cdot 10^{-6}$ M A23187 does not induce chemiluminescence with granulocytes from patients with chronic granulomatous disease even though depolarization occurs.

Discussion

Granulocytes from patients with chronic granulomatous disease do not exhibit a 'respiratory burst' in response to particle-stimulation [4]. This failure to release active forms of oxygen would explain the inability of these diseased cells to kill bacteria [17]. It has, therefore, been concluded that the defect in chronic granulomatous disease resides in the oxidase [7].

Various stimulants induce shifts in the transmembrane potential of normal granulocytes which precede and may trigger the 'respiratory burst' [8–11]. *N*-Formyl-methionyl-leucyl-phenylalanine, concanavalin A, and phorbol myristate acetate induce a biphasic potential change, i.e., a rapid depolarization followed by a prolonged hyperpolarization [8–11]. In contrast, the calcium ionophore (A23187) induces a monophasic potential change, i.e., a rapid and prolonged depolarization [9,18]. Recent reports indicate that stimulant-induced biphasic shifts in transmembrane potential are absent in granulocytes from patients with chronic granulomatous disease [10,18]. Unfortunately, these authors suggest that the delayed hyperpolarization or the initial depolarization may be an artifact in their respective systems [10,18]. Therefore, we attempted to verify these results using Di-S-C_3(5) as a probe. We used several stimulants which induce the generation of chemiluminescence or superoxide release in normal granulocytes. All of these stimulants fail to activate the 'respiratory burst' in granulocytes from a patient with chronic granulomatous disease. Three of these stimulants, i.e., *N*-formyl-methionyl-leucyl-phenylalanine, concanavalin A, and phorbol myristate, also fail to perturb the transmembrane potential of diseased granulocytes. In contrast, A23187 induces a normal depolarization even in diseased cells, yet no superoxide release occurs.

N-Formyl-methionyl-leucyl-phenylalanine, concanavalin A, and phorbol myristate acetate presumably stimulate granulocytes by binding to membrane receptors. It has been suggested that in normal granulocytes this stimulant-receptor interaction results in an increase in sodium and calcium permeability and thus, membrane depolarization [18,19,21,22,8,9]. It has been shown that stimulant-receptor binding is not greatly decreased in chronic granulomatous disease [18]. Therefore, for these three stimulants, the defect in chronic granulomatous disease may be failure of stimulant-recptor interaction to initiate normal changes in membrane permeability. Thus, no shifts in membrane potential would occur. Without this electrical signal, superoxide release would not be initiated. In contrast, A23187 depolarizes granulocytes directly, i.e., by forming calcium channels in the membrane, and does not require interaction with membrane receptors. Therefore, one would expect A23187 to depolarize diseased granulocytes even if a defect exists in the receptor triggering mechanism. The fact that A23187 fails to induce superoxide release from diseased granulocytes suggests that a second defect must also exist which prevents depolarization from resulting in superoxide release. This defect may indeed reside in the oxidase as proposed by Allen et al. [7].

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