Use of Lipophilic Probes of Membrane Potential to Assess Human Neutrophil Activation

ABNORMALITY IN CHRONIC GRANULOMATOUS DISEASE

BRUCE E. SELIGMANN and JOHN I. GALLIN, *Bacterial Disease Section, Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205*

ABSTRACT Previous studies using membrane potential sensitive probes have provided evidence that chemotactic factors elicit membrane potential changes in normal human neutrophils (PMN). In addition to stimulation of PMN motility, chemotactic factors also stimulate degranulation and superoxide ion (O_2^-) generation and it has been suggested that alteration of membrane potential activates these events (Korchak, H. M., and G. Weissmann. 1978. *Proc. Natl. Acad. Sci. U. S. A.* **75**: 3818–3822.). To further define the interrelationship of these functions, studies were done with two indirect probes of membrane potential, 3-3′-dipentyloxacarbocyanine and triphenylmethylphosphonium ion ($TPMP^+$) using PMN from normal subjects, from patients with abnormal O_2^- production (chronic granulomatous disease [CGD]), and from patients with defective degranulation and/or chemotaxis (Chediak-Higashi syndrome and patients with elevated immunoglobulin (Ig)E and recurrent staphylococcal infections). The stimuli used were the chemoattractant *N*-formyl-methionyl-leucyl-phenylalanine (f-Met-Leu-Phe) and the secretagogues ionophore A23187 and phorbol myristate acetate (PMA). The results obtained with 3-3′-dipentyloxacarbocyanine and $TPMP^+$ were comparable. The apparent membrane potential changes elicited by f-Met-Leu-Phe and PMA in normal PMN were reduced or entirely absent in PMN obtained from patients with CGD but normal in PMN from other patients. PMN from patients with CGD had normal calculated resting membrane potentials and normal responses elicited by the potassium ionophore valinomycin. The responses to calcium ionophore A23187 were only slightly impaired. The abnormality of the elicited response of CGD cells to f-Met-Leu-Phe and PMA could not be attributed to the absence of O_2^-, hydroxyl radical, singlet oxygen, or hydrogen peroxide acting on the probes. Instead this abnormality appears to be associated with a dysfunction in the normal molecular mechanism(s) stimulated upon neutrophil activation. The data suggest chemoattractant alteration of membrane potential in normal PMN is related to activation of oxidative metabolism but the relationship to chemotaxis and degranulation remains to be established.

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INTRODUCTION

A diverse group of agents such as chemoattractants, the croton oil derivative phorbol myristate acetate (PMA),[1] and the calcium ionophore A23187 stimulate superoxide generation in human neutrophils (polymorphonuclear leukocytes [PMN]) (2–6). The failure of PMN to generate superoxide ion when stimulated with these agents has been correlated with reduced killing of catalase-positive microorganisms and is characteristic of patients with hereditary deficiency of oxidative metabolism or chronic granulomatous disease (CGD) of childhood (7–12).

The cellular mechanism leading to the production of superoxide, secretion, or chemotaxis is not known, nor is the cause of the defect in PMN from patients with CGD. However, it is generally accepted that activation of a plasma membrane NADP(H) oxidase is involved in the production of superoxide (13–17). In addition, studies with a variety of stimulants of superoxide generation, particularly chemoattractants, have indicated that these stimuli all initiate changes in leu-

[1] *Abbreviations used in this paper*: CDG, chronic granulomatous disease; di-O-C_5(3), 3-3′-dipentyloxacarbocyanine; PMA, phorbol myrisate acetate; PMN, polymorphonuclear leukocyte; TPB, tetraphenylboron; $TPMP^+$, triphenylmethylphosphonium ion.

kocyte calcium, sodium, and potassium fluxes (2, 18–23) and alteration of leukocyte membrane electrochemical potential (referred to in this paper as membrane potential) (24–30). Korchak and Weissmann (24), using the indirect probe of membrane potential triphenylmethylphosphonium ion ($TPMP^+$), have reported that stimulus-induced alteration of the plasma membrane potential precedes superoxide generation in neutrophils. They suggested that changes in membrane potential represent an early step in the sequence of events leading to superoxide generation (24, 28). In other studies Lehrer (31) reported that neutrophils from patients with CGD have low rubidium efflux after phagocytosis, which the authors suggested was closely related to the abnormality of oxidative metabolism. In this study two indirect probes of membrane potential, the fluorescent cyanine dye 3-3′-dipentyloxacarbocyanine [di-O-C_5(3)]and the radiolabeled ion $TPMP^+$, were used to study the possibility of an abnormality of elicited membrane potential response in neutrophils obtained from patients with CGD.

METHODS

Materials. Assays were carried out using phosphate-buffered Hanks' solutions containing 128.5 mM NaCl, 4.2 mM KCl, 0.9 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 0.4 mM $MgSO_4$, 1 mM $MgCl_2$, 1.6 mM $CaCl_2$, 5 mM glucose, and 5 mM $NaHCO_3$. For some experiments the extracellular concentration of potassium was varied using solutions of Hepes- (Sigma Chemical Co., St. Louis, Mo.) buffered Hanks' solutions, containing 10 mM NaCl, 0.9 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 0.2 mM $MgSO_4$, 0.1 mM $MgCl_2$, 1.6 mM $CaCl_2$, 11 mM glucose, and 10 mM Hepes (pH 7.3) and varied amounts of KCl and choline chloride. The concentration of potassium was varied by reciprocally adjusting the concentration of potassium chloride and choline chloride to give the appropriate final concentration of potassium while maintaining the osmolarity and concentration of all other ions.

The calcium ionophore A23187 (provided by Robert Hamill, Eli Lilly and Co., Indianapolis, Ind.), the croton oil derivative PMA (Consolidated Midland Corp., Brewster, N. Y.), cytochalasin B (Aldrich Chemical Co., Inc., Milwaukee, Wis.), *N*-formyl-methionyl-leucyl-phenylalanine (f-Met-Leu-Phe; provided by Elliot Schiffmann, National Institute for Dental Research, National Institutes of Health), and valinomycin (Sigma Chemical Co.) were all dissolved in dimethyl sulfoxide (Fisher Scientific Co., Pittsburgh, Pa.) to make appropriate stock solutions such that the final concentration of dimethyl sulfoxide in the reaction mixture was 0.1%. This amount of dimethyl sulfoxide did not have any effect on secretion, superoxide generation, cell viability as measured by trypan blue exclusion and lactate dehydrogenase release, or the fluorescence response.

Obtained as follows were: Hypaque (Winthrop Laboratories, New York), Ficoll and T250 dextran (Pharmacia, Uppsala, Sweden), tritiated $TPMP^+$, [^{14}C]urea, and [^{14}C]-inulin (New England Nuclear, Boston, Mass.), unlabeled $TPMP^+$ (Pfaltz & Bauer, Inc., Stamford, Conn.), tetraphenylboron (TPB, Sigma Chemical Co.), Versilube F50 (Harwick Chemical Corp., Trenton, N. J.), NCS tissue solubilizer (Amersham Corp., Arlington Heights, Ill.), 3a 20 counting solution (Research Products International Corp., Elk Grove Village, Ill.), superoxide dismutase (Miles Laboratories, Inc., Elkhart, Ind.), xanthine, xanthine oxidase, and cytochrome *c* (Sigma Chemical Co.).

Patients. Six males and one female ranging in age from 2.5–21 yr with CGD were studied. They are referred to throughout the text by their initials. Three of the patients [C.H. (the female), B.P., and P.R.] were reported previously (32–34). When stimulated with the chemoattractant f-Met-Leu-Phe, the calcium ionophore A23187, or the croton oil derivative PMA, neutrophils from each patient exhibited a deficiency of oxidative metabolism as assessed by nitroblue tetrazolium dye reduction (35). PMN from these patients also failed to generate superoxide (superoxide dismutase-inhibitable cytochrome *c* reduction assay; see below). None of the patients were infected or on antibiotics at the time of study. For some studies PMN from two brothers with the Chediak-Higashi syndrome (36) and three patients with abnormal PMN chemotaxis, markedly elevated immunoglobulin (Ig)E, and recurrent skin and pulmonary infections were studied (37).

Leukocyte isolation. Peripheral blood PMN from normal human subjects were prepared by Ficoll-Hypaque gradient centrifugation followed by dextran sedimentation (38). This technique routinely resulted in cell populations containing over 95% PMN. All experiments were completed within 6 h of obtaining the cells unless otherwise indicated. To minimize cell aggregation, PMN were kept on ice in calcium and magnesium-free Hanks' solution for up to 3 h until use. Before each study the PMN were incubated at 37°C for 10 min in divalent cation containing medium. The cells had an additional 5-min equilibrium at 37°C in the cuvettes during equilibration with the probe.

Functional studies. Superoxide was generated using xanthine (0.1 mM) and xanthine oxidase (0.05 U/ml) or intact neutrophils using appropriate stimuli. The presence of superoxide was determined spectrophotometrically (549 nm, extinction coefficient of 21.1) by continuously monitoring the superoxide dismutase (3 U/ml) inhibitable reduction of cytochrome *c* (120 μg/ml) by a stirred suspension of cells, a modification of techniques described previously (39–41). Using these conditions 50 μmol/min O_2^- were generated, approximately 10 times the amount of O_2^- produced by normal neutrophils after stimulation with PMA (50 ng/ml).

To relate the observations obtained using the probes of membrane potential to other cell functions, migration and degranulation were also assayed. PMN migration was quantitated using two micropore filter techniques. For the first method the distribution of a population of PMN in a 3.0-μm cellulose nitrate filter (Sartorius, Testing Machines Inc., Amityville, N. Y.) was determined after a 45-min incubation (37°C) according to the method of Zigmond and Hirsch (42). To facilitate counting of the filters, a Zeiss photomicroscope 2 (Carl Zeiss, Inc., New York) was connected to an image analyzer (Optomax CPU-2, Micromeasurements, Cambridge, England) interfaced with a Hewlett-Packard 9815 calculator and 7225A plotter (Hewlett-Packard Co., Palo Alto, Calif.). With this system two 73-μm^2 cores in each of four micropore filters were assessed with measurements made every 10 μm into the filters. The mean distance migrated was determined as described by Maderazo et al. (43). In the second assay of migration a radioassay employing ^{51}Cr cells and a double micropore filter system was used (44). For the latter assay data were expressed as the percent migration of PMN from a normal subject run concurrently. The stimuli of locomotion were Gey's buffer, *Escherichia coli* endotoxin (Difco Laboratories, Detroit, Mich.) activated human serum (5% vol/vol) and f-Met-Leu-Phe (10 nM). In some studies the effect of serum from patients with CGD on normal PMN migration was assessed by

incubating sera from normal controls or patients with CGD with normal PMN (3% vol/vol) in Gey's media for 30 min at 37°C. Chemotaxis of these serum-treated cells was assessed using the radioassay of PMN migration and *E. coli* endotoxin-activated serum (5% vol/vol) or f-Met-Leu-Phe (10 nM) as the stimuli.

The ability of PMN to release their lysosomal granule contents in response to the stimuli ionophore PMA (20 ng/ml), A23187 (0.1 μM), or f-Met-Leu-Phe (1 μM) plus cytochalasin B (5 μg/ml) was also determined. PMN (5×10^6 cells/ml in Hanks' solution) were incubated with the various stimuli for varying times at 37°C and centrifuged. The release of lysozyme or β-glucuronidase into the extracellular (supernatant) fluid was determined and compared with the total cellular enzyme as described previously (45).

Reconstitution of PMN from CGD patients with superoxide-generating systems. Two approaches were used to "reconstitute" PMN from patients with CGD. One method was to incubate neutrophils (2.5×10^6 PMN/ml) with xanthine (0.1 mM) plus xanthine oxidase (0.05 U/ml). The second approach was to incubate PMN from patients with CGD with superoxide-generating (catalase negative) bacteria. A rough strain of opsonized (AB serum) *Streptococcus pneumoniae* (type 2, courtesy of Dr. Stephen Hosea, National Institutes of Health) was used at a ratio of 50 bacteria to 1 PMN as described (46) in the presence of 10% serum at 37°C for 45 min. After four washes to remove bacteria not associated with the PMN, fluorescence studies with di-O-C_5(3) were performed. Under these conditions the PMN from patients with CGD killed the *S. pneumoniae* normally.

General procedure for the fluorometric assay of membrane potential. The cyanine dye di-O-C_5(3) was a generous gift from Dr. Alan Waggoner (Amherst College, Amherst, Mass.). Stock solutions of dye (1 mM) were made in ethanol and kept in the dark at 4°C. Before each set of experiments this stock was diluted 100-fold into Hanks' solution to make a working solution that was discarded after 4 h of use.

The details of the assay have been reported recently (30). A standard assay volume of 1 ml Hanks' solution was used in a 1-cm pathlength cuvette. To this 2.5 μl of the working dye solution was added, giving an initial dye concentration of 0.25 μM and <0.05% ethanol (this amount of ethanol had no effect on cell viability or fluorescence). The fluorescence was recorded using a Farrand Mark I spectrofluorometer (Farrand Optical Co., Inc., Valhalla, N. Y.) with the excitation wavelength set at 460 nm and the fluorescence wavelength at 510 nm. The temperature of the cuvette was maintained at 37°C, and the cell suspension was maintained by means of a magnetic flea and stirrer (300 rpm).

As noted by others (47), approximately two-thirds of the dye adsorbed onto the cuvette and stirring flea, causing the fluorescence to decrease to a constant value within 4 min. Once the fluorescence had stabilized, 75 μl of a suspension of PMN was added to give a final concentration of 2.5×10^5 cells/ml. The uptake of dye by the PMN resulted in a change in fluorescence as described in Results. The mechanism of dye fluorescence when associated with PMN has been described (30). Unlike other cyanine dyes, di-O-C_5(3) does not aggregate readily. Under the conditions used in the current studies, dye aggregation was avoided (30) and thus the uptake of dye by PMN resulted in enhancement rather than the quenching of fluorescence seen with other cyanine dyes. The level of fluorescence of equilibrated unstimulated cells appears to reflect the electrochemical membrane potential of the PMN and is reduced by increasing the extracellular concentration of potassium in a manner predicted for membrane potential by the Goldman equation (30). Lysis of cells results in loss of fluorescence and treatment with ionophores causes changes in fluorescence consistent with their predicted effect on membrane potential. In addition, it has been shown with butanol extraction studies that the amount of dye accumulated by cells is proportional to the measured fluorescence (30). In this paper the fluorescence changes are reported as a percentage of the total full-scale fluorescence. During these experiments the photomultiplier tube sensitivity was kept constant, permitting quantitative comparison of results. Cell concentration, viability, and aggregation were determined microscopically at the end of each experiment using trypan blue and phase-contrast microscopy.

General procedure for $TPMP^+$ assay of membrane potential. In previous studies employing $TPMP^+$ as a probe of membrane potential up to 3 h were needed for the $TPMP^+$ to equilibrate with the PMN (24). In preliminary studies, using only $TPMP^+$, we were able to confirm Korchak and Weissmann's recent report (28) on the effect f-Met-Leu-Phe has on the distribution of this probe in PMN (transient accumulation followed by loss and then reaccumulation). However this response was not always obtained. In some instances the initial transient accumulation of probe was not observed. The lack of consistency appeared to be a result of the 3 h incubation required in our experiments for equilibration of the $TPMP^+$ with PMN before exposure to the stimulus. Therefore we modified the $TPMP^+$ assay by adding TPB, which Deutsch et al. (48) reported increases the rate of $TPMP^+$ equilibration without effecting the final $TPMP^+$ equilibrium value in erythrocytes and lymphocytes. TPB did not effect PMN viability, chemotactic responsiveness, ability to generate superoxide anion, or the membrane potential changes monitored using the cyanine dye di-O-C_5(3). TPB also had no effect on the membrane potential calculated from the distribution of $TPMP^+$ (49).

For the $TPMP^+$/TPB assay PMN (5×10^6 cells/ml, Hanks' solution) were incubated for 5 min with 10 μM TPB in a shaking water bath at 37°C. Tritiated $TPMP^+$ (final concentration 50 μM, specific activity of 15 μCi/mmol and either [^{14}C]urea (57 mCi/mmol sp act) or [^{14}C]inulin (5 mCi/mmol sp act) were added after the 5-min incubation. Samples (300 μl) were withdrawn periodically and layered on top of heated silicone oil (37°C) contained in a 1.5 ml Eppindorf microfuge tube (500 μl of Versilube F50 at 37°C). The samples were centrifuged for 10 s, a sufficient amount of time for the cells to be pelleted at the bottom of the tube. The aqueous phase and silicone oil were aspirated and the tip of the centrifuge tube, containing the cell pellet, was cut off into a scintillation vial to which 1 ml of toluene-based tissue solubilizer (NCS) was added. The vials were shaken overnight to thoroughly dissolve the cell pellet and then 5 ml of a toluene-based scintillation fluid was added. The samples were allowed to dark adapt for 1 h before counting ^{14}C and ^{3}H using a Beckman LS9000 scintillation counter with automatic quench compensation (Beckman Instruments, Inc., Fullerton, Calif.). The extracellular trapped space was determined in a separate series of experiments using [^{14}C]-inulin and tritiated urea. This space was highly reproducible and small relative to the total urea space (<6%).

Each time point value was determined in triplicate and results expressed either as a calculated membrane potential, $\Psi \pm$SEM or as a corrected calculated membrane potential, Ψ_{corr}. Membrane potential was calculated using the following equation, where R is the molar gas constant, T is the temperature in degrees Kelvin, and F is a Faraday. The remaining values were measured as follows: [TPMP inside] as ^{3}H counts per minute in the cell pellet, [UREA inside] as ^{14}C counts per minute in the cell pellet, [TPMP outside] as ^{3}H counts per minute/10 μl supernate, and [UREA outside] as ^{14}C counts per minute/10μl supernate.

$$\Psi = -\frac{RT}{F} \ln \left(\frac{[\text{TPMP}^+ \text{ inside}][\text{UREA outside}]}{[\text{UREA inside}][\text{TPMP}^+ \text{ outside}]} \right) \quad (1)$$

Cell viability (trypan blue exclusion and lactate dehydrogenase release) and in some instances di-O-C_5(3) fluorescence were monitored concurrently. The distribution of $TPMP^+$ was sensitive to potassium gradients, similar to di-O-C_5(3) fluorescence. Fig. 1 shows the effect of varying extracellular potassium on $TPMP^+$ accumulation by cells expressed as the calculated membrane potential Ψ Eq. 1. The experiment shown demonstrates that accumulation of $TPMP^+$ is apparently not only sensitive to membrane potential but also contains a membrane potential insensitive component. In the presence of 120 mM external potassium (conditions under which the cells would be expected to be fully depolarized), the membrane potential calculated using Eq. 1 was −30 mV instead of the predicted value of 0 mV for complete depolarization. This difference between the observed and expected membrane potential may be attributable to nonspecific (residual) binding of $TPMP^+$ (48–50) and is defined as $TPMP_R$. Accordingly, corrected values for membrane potential (Ψ_{corr}) were then calculated using this additional correction by the following equation:

$$\Psi_{corr} = -\frac{RT}{F} \ln \left(\frac{[\text{TPMP inside}] - [\text{TPMP}_R]}{[\text{UREA inside}]} \times \frac{[\text{UREA outside}]}{[\text{TPMP outside}]} \right) \quad (2)$$

Since the Ψ_{corr} values are based on the assumption that in the presence of 120 mM external potassium PMN are completely depolarized and since presently there is no means by which this can be confirmed using intracellular recording, these values represent only an estimate of the actual membrane potential.

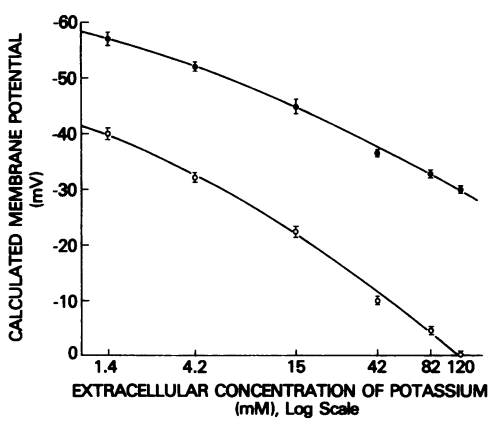

FIGURE 1 Effect of external potassium on $TPMP^+$ distribution. The membrane potentials calculated from $TPMP^+$ distribution data using Eq. 1 (Methods) are plotted as a function of the log extracellular potassium concentration (●). Similarly corrected values calculated using Eq. 2 (Methods) are plotted (○). In these experiments sodium was kept at 10 mM, while potassium was varied by substitution with choline chloride. Each point represents the mean±SEM of three observations made at equilibrium (30 min incubation with $TPMP^+$ plus TPB).

RESULTS

Studies using di-O-C_5(3)

Effect of the chemoattractant f-Met-Leu-Phe. As shown in Fig. 2, addition of normal PMN to a solution containing the fluorescent cyanine dye di-O-C_5(3) (0.25 μM) resulted in an enhancement of fluorescence as dye partitioned into the cells. The final steady-state value of fluorescence reflects the resting membrane potential (30). The addition of the chemoattractant f-Met-Leu-Phe caused a biphasic response with a decrease followed by an increase in fluorescence. The response was dose dependent over a range of 1 nM to 1 mM peptide, with 0.1 μM f-Met-Leu-Phe producing a maximal response (30). When various synthetic peptides were used, the dose giving a half maximal response corresponded to the relative efficacy of the peptides as chemoattractants (30). Based on ion substitution studies reported (27, 29, 30), the first phase of the response (decrease in fluorescence) could not be conclusively attributed to a change in membrane potential (depolarization) across the plasma membrane (30). For example, it could also reflect potential changes across intracellular organelles. In addition, it is possible that the first component of the fluorescence response reflects PMN heterogeneity or represents a summation of responses of individual cells within the population over a period of time. If the latter were the case, some cells could be hyperpolarizing (repolarizing) at the same time other cells were just beginning to depolarize. Thus, for these reasons we have concluded that although the response is compatible with a depolarization, this conclusion is not unequivocal (30). The second phase or increase in fluorescence, however, has been more readily defined and is consistent with

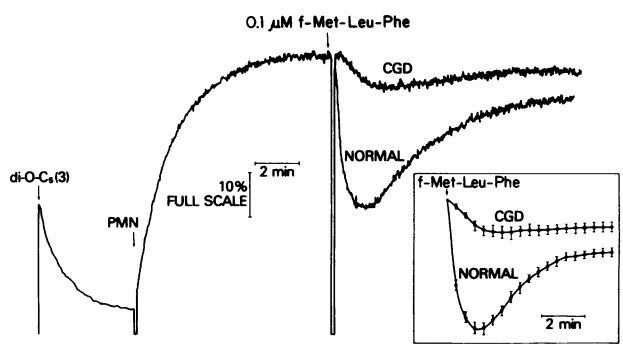

FIGURE 2 The effect of f-Met-Leu-Phe (0.1 μM) on di-O-C_5(3) fluorescence of PMN from a normal control (lower tracing) and a patient with CGD (upper tracing). Time scale, percent full-scale fluorescence, and addition of di-O-C_5(3), PMN, and f-Met-Leu-Phe are indicated. The spikes seen upon each addition are artifact caused by the opening and closing of the photomultiplier tube. The inset shows the mean±SEM fluorescence for six patients with CGD and six controls.

a hyperpolarization of the plasma membrane resulting from an increase in plasma membrane permeability to potassium (30).

PMN obtained from each of six patients with CGD had an abnormal fluorescence response elicited by f-Met-Leu-Phe. The inset (Fig. 2) shows pooled data from six patients and six controls run concurrently. The basal di-O-C_5(3) fluorescence (resting membrane potential) of unstimulated CGD neutrophils was normal. Responses shown were elicited with 0.1 μM f-Met-Leu-Phe and represent the maximum response; 1,000-fold higher concentrations of peptide did not elicit any larger response. The abnormal response of PMN from patients with CGD was characterized as a reduced first phase (apparent depolarization) and an absent second phase (hyperpolarization or repolarization).

Effect of valinomycin, A23187, and PMA. To assess whether the plasma membrane of CGD cells responded normally to changes in ion permeability, the effect of the potassium ionophore valinomycin was assessed. In prior studies we showed that valinomycin produced a potassium-dependent increase in di-O-C_5(3) fluorescence (compatible with a membrane hyperpolarization [30]). As shown in Fig. 3, the response of neutrophils from three patients with CGD to valinomycin (1 μM) was normal.

Other experiments were performed with the calcium ionophore A23187 and PMA, both of which generate superoxide from PMN but by apparently different mechanisms (21, 51). As shown in Fig. 4, high concentrations of these stimuli caused a decrease in

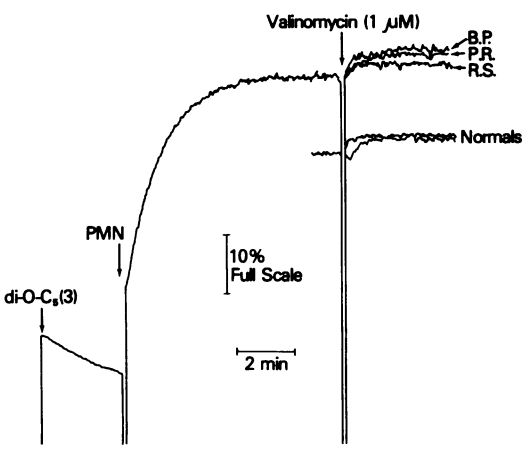

FIGURE 3 Effect of the potassium ionophore valinomycin (1 μM) on di-O-C_5(3) fluorescence of PMN obtained from two normal subjects and three patients with CGD (B.P., P.R., and R.S.). Time scale, percent full-scale fluorescence, and addition of di-O-C_5(3), PMN, and valinomycin are indicated for the upper tracings. For clarity the lower tracing is offset and only 1 min of the fluorescence recording before addition of valinomycin is shown. The absolute level of fluorescence before addition of stimulus was the same in both tracings.

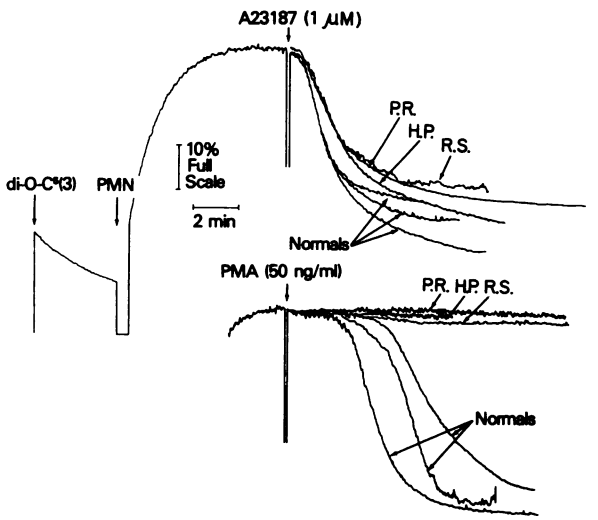

FIGURE 4 Effect of the calcium ionophore A23187 (1 μM) and PMA (50 ng/ml) on di-O-C_5(3) fluorescence of PMN obtained from three normal subjects and three patients with CGD. Time scale, percent full-scale fluorescence, and addition of di-O-C_5(3), PMN, and stimuli are indicated. For clarity the lower tracing is offset and only 2 min of the fluorescence recording before addition of PMA is shown; the absolute level of fluorescence before addition of stimuli was the same as in the upper tracings.

fluorescence compatible with a membrane depolarization. A difference between the response of normal PMN to A23187 and PMA was the delayed onset of the PMA-stimulated response. As shown in Fig. 4 (upper tracings), compared to normal cells, neutrophils from patients with CGD gave an essentially normal response to A23187 (1 μM), although the magnitude of the response was significantly smaller ($P < 0.05$, paired sample t test). In contrast to A23187, PMA failed to give any response to CGD cells (Fig. 4, lower tracings).

Effect of neutrophil products on di-O-C_5(3) fluorescence. Since PMN from patients with CGD do not generate superoxide, the possibility was investigated that superoxide or other reactive species such as hydroxyl radical, singlet oxygen or hydrogen peroxide have a direct effect on di-O-C_5(3) fluorescence. When superoxide was generated by xanthine plus xanthine oxidase in the presence of di-O-C_5(3), there was no effect on fluorescence either with or without cells. Incubation of normal PMN with xanthine plus xanthine oxidase and subsequent challenge with f-Met-Leu-Phe resulted in a normal fluorescence response (Fig. 5). Generation of superoxide ion by xanthine plus xanthine oxidase in the presence of PMN obtained from patients with CGD did not modify the abnormal responses to PMA or f-Met-Leu-Phe. Furthermore, ingestion of the catalase negative (superoxide generating) bacteria *S. pneumonia* (Methods) had no effect on

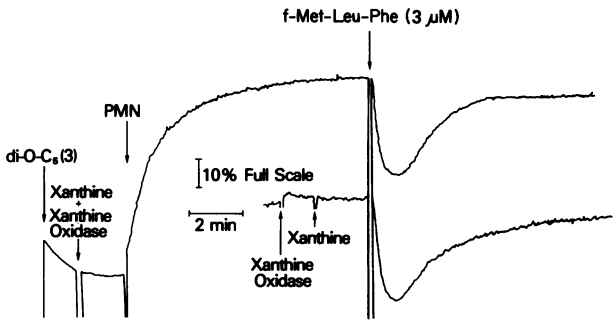

FIGURE 5 Effect of xanthine plus xanthine oxidase on the equilibration of PMN with di-O-C_5(3) and the subsequent fluorescence response to f-Met-Leu-Phe. Upper tracing shows addition of xanthine plus xanthine oxidase to di-O-C_5(3) before PMN, and lower tracing shows addition of xanthine plus xanthine oxidase after di-O-C_5(3) PMN equilibration. Time scale, percent full-scale fluorescence, and the addition of di-O-C_5(3), xanthine, xanthine oxidase, PMN, and f-Met-Leu-Phe are indicated.

subsequent PMA or f-Met-Leu-Phe responses of normal PMN or cells obtained from patients with CGD.

In related studies, addition of catalase (110 U/ml) (which degrades hydrogen peroxide) or superoxide dismutase (3 U/ml) (which degrades superoxide anion) (5) to normal PMN 5 min before addition of f-Met-Leu-Phe had no effect on the elicited response. Similarly incubation of PMN for 5 min with azide (1 mM, an inhibitor of myeloperoxidase and singlet oxygen formation), mannitol (20 mM, scavenger of hydroxyl radical), histidine (0.1 mM, scavenger of singlet oxygen), and cysteamine (10 mM, cell permeable scavenger of superoxide [6, 52–55]) had no effect on basal di-O-C_5(3) fluorescence or the response elicited by f-Met-Leu-Phe (0.1 μM). Similarly cyanide (2 mM), an inhibitor of myeloperoxidase (55), did not effect the response to f-Met-Leu-Phe, although this high concentration of cyanide did cause a gradual decrease in basal fluorescence after a 10–20 min incubation.

Other studies were designed to assess the possible effect of neutrophil products on di-O-C_5(3) fluorescence. The addition of 50 μl of a cell preparation lysed with distilled water and repeated freezing and thawing (2.5×10^5 cells) had no effect on the resting fluorescence of PMN or on the fluorescence response elicited by f-Met-Leu-Phe. In additional experiments, designed to assess the possible effect small molecular weight neutrophil-secreted products might have on fluorescence, di-O-C_5(3) was contained in a dialysis membrane with PMN outside. Preliminary studies indicated that the dye diffused only very slowly across the dialysis membrane, and thus the membrane acted as an effective barrier to separate dye from the cells. In this system, when the PMN were stimulated with addition of either f-Met-Leu-Phe (1 μm) or the calcium ionophore A23187 (1 μM) to cells outside the membrane no change in fluorescence was observed, indicating secreted neutrophil products crossing the dialysis membrane do not alter di-O-C_5(3) fluorescence. In a control experiment it was confirmed that the dialysis membrane had no adverse effect on the dye and did not mask the elicited response. Thus the abnormality seen in CGD cells could not be related to lack of small molecular weight products acting on di-O-C_5(3).

Studies using neutrophils from patients with other phagocyte defects. PMN from two patients with the Chediak-Higashi syndrome (Fig. 6) and three patients with abnormal PMN chemotaxis, markedly elevated IgE and recurrent skin and pulmonary infections with *Staphylococcus aureus* were studied. The fluorescence responses of PMN from patients with high IgE and recurrent infection were completely normal. The response to f-Met-Leu-Phe of the PMN from the Chediak-Higashi patients was also essentially normal except that the time-course of the response was prolonged (Fig. 6). In addition, as shown in Fig. 6, the basal fluorescence of La.R. consistently (four studies) drifted from the initial basal state, although the response elicited by f-Met-Leu-Phe was normal.

Studies using TPMP⁺/TPB

The average resting membrane potential of PMN from five normal subjects was −45±2 mV. This is ~20 mV more negative than the PMN resting membrane potential reported using $TPMP^+$ in the absence of TPB (24). Control experiments indicated that these differences were due to both the shorter incubation time required for $TPMP^+$ equilibration in the presence of TPB, avoiding adverse effects of cell aging on mem-

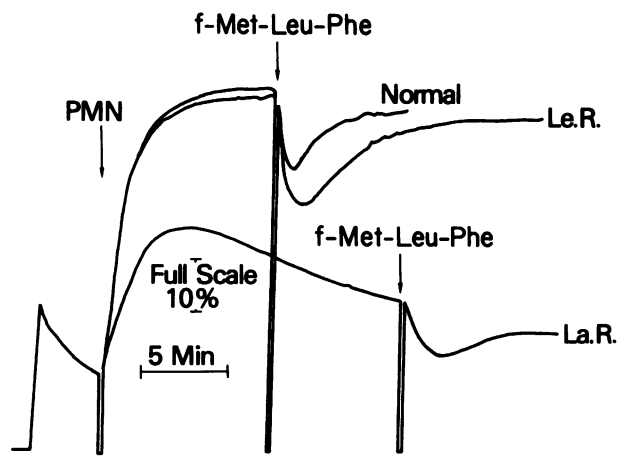

FIGURE 6 Effect of f-Met-Leu-Phe (0.1 μM) on di-O-C_5(3) fluorescence of PMN from a normal subject and two patients with the Chediak-Higashi syndrome (Le.R. and La.R.). Time scale, percent full-scale fluorescence, and addition of di-O-C_5(3), PMN, and f-Met-Leu-Phe are indicated.

brane potential (49), and to the corrections made for nonspecific $TPMP^+$ binding (Eq. 2). The average resting membrane potential of PMN from three patients with CGD was normal (−44.9±2.9 mV). Stimulation of $TPMP^+$/TPB equilibrated normal control cells with f-Met-Leu-Phe (0.1 μM) or PMA (20 ng/ml) caused a loss of $TPMP^+$ followed by its reaccumulation (Fig. 7). In contrast, both f-Met-Leu-Phe and PMA had a negligible effect on $TPMP^+$ distribution in PMN from patients with CGD.

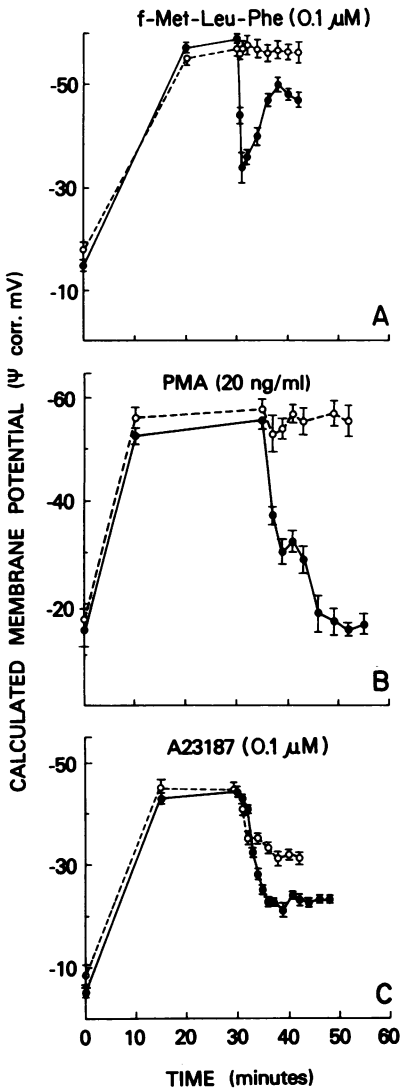

FIGURE 7 Membrane potential changes in CGD PMN assessed with $TPMP^+$. The point of addition of each stimulus was at 30 min. A: Effect of f-Met-Leu-Phe (0.1 μM) on the distribution of $TPMP^+$. B: Effect of PMA (20 ng/ml) on the distribution of $TPMP^+$. C: Effect of the calcium ionophore A23187 (0.1 μM) on the distribution of $TPMP^+$. All three panels represent data obtained in a single experiment using PMN from the same normal subject (●) and a patient (P.R.) with CGD (○). Mean±SEM, triplicate samples.

Since the di-O-C_5(3) fluorescence response of PMN from CGD patients after stimulation with the calcium ionophore A23187 was minimally abnormal, the effect of A23187 on $TPMP^+$ distribution in cells from both normal and CGD subjects was also investigated (Fig. 7). Although a response was elicited with A23187 from the PMN of patients with CGD, the magnitude of the response was significantly ($P < 0.02$, paired sample t test) less than normal, similar to the data obtained with di-O-C_5(3) (Fig. 4).

Functional studies

Studies of PMN locomotion and degranulation were performed using PMN from patients with CGD in an effort to further define the relationship between PMN function and responses seen with the membrane potential sensitive probes. Using a morphologic assay of neutrophil locomotion the migration in response to buffer (random migration) was normal (Table I and Fig. 8, $P > 0.05$). However, a small yet significant defect in the chemotaxis response to both *E. coli* endotoxin-activated sera (62% normal) and f-Met-Leu-Phe (64% normal) was seen in neutrophils from patients with CGD. Similar data were obtained in three patients using a ^{51}Cr radioassay of neutrophil locomotion. In two related studies, designed to test the possibility that sera from CGD subjects contained an inhibitor of chemotaxis, serum (3% vol/vol) obtained from five patients with CGD was incubated with PMN obtained from two different normal subjects. Such treatment had no effect on the subsequent locomotory response of the normal cells to *E. coli* endotoxin-activated serum (5% vol/vol) or f-Met-Leu-Phe (10 nM), indicating that in our CGD patients the abnormality of chemotaxis could not be related to a serum factor interacting with the PMN.

Degranulation was also studied in PMN obtained from four patients. PMN from each subject were incubated for varying time periods (2–30 min) with the secretagogues PMA (20 ng/ml), A23187 (0.1 μM), or f-Met-Leu-Phe (1 μM) plus cytochalasin B (5 μg/ml), or with buffer. In two subjects (P.R. and E.H.) the percentage of total cellular lysozyme released was significantly lowered ($P < 0.05$) at 2 and 5 min after addition of each stimulus but normal after 15 min. However, in two subjects (E.H. and B.P.) there was no abnormality detected at any of the time points.

DISCUSSION

The use of two different lipophilic probes of membrane potential has provided evidence that neutrophils (24, 26–30) and monocytes (56) respond to chemoattractants and other stimuli with alteration of membrane potential. The responses are qualitatively similar to the membrane potential changes recorded

TABLE I
Locomotion of Neutrophils Obtained from Subjects with CGD

Stimulus / Subject	Neutrophil locomotion	
	Normal	CGD
	*μM**	
Buffer		
C.H.	17	14
P.R.	12	10
B.P.	8	15
E.H.	5	14
S.S.	—	16
H.P.	15	10
D.F.	22	9
	13.2±2.5	12.6±1.1
E. coli endotoxin-activated serum, 5% vol/vol		
C.H.	41	43
P.R.	39	23
B.P.	53	20
E.H.	33	22
E.H.	—	25
H.P.	45	24
	42.2±3.3	26.2±3.4‡
f-Met-Leu-Phe, 10 nM		
B.P.	31	18
E.H.	33	27
S.S.	29	17
D.F.	34	27
A.G.	41	18
	33.6±2.0	21.4±2.3‡

* Mean±SEM average distance migrated by a population of neutrophils into a 3.0-μM cellulose nitrate filter in response to the indicated stimulus for two readings in each of four replicate filters. (Methods)

‡ Significance of the level of difference between normal and CGD, Student's *t* test.

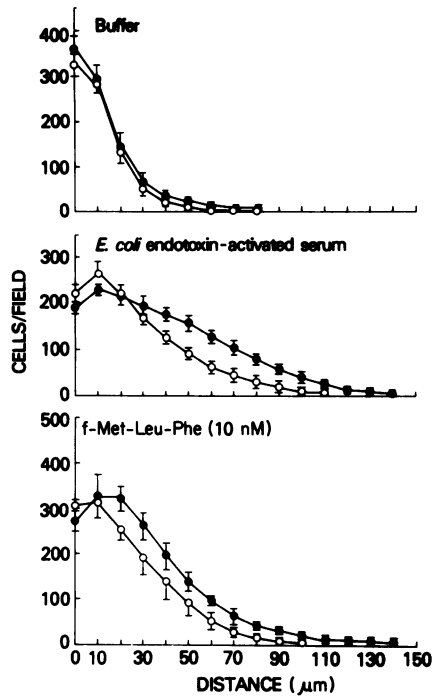

FIGURE 8 Migration of normal and CGD PMN into a micropore filter during a 45 min incubation in response to Gey's buffer, 5% (vol/vol) *E. coli* endotoxin-activated serum or f-Met-Leu-Phe (10 nM). Results are expressed as the number of cells in a 73 μm^2 field at the indicated distance in the filter. Each point represents the mean±SEM of data obtained from studies using PMN from five different normal subjects or five patients with CGD.

from cultured macrophages using direct intracellular recording techniques (25, 56, 57). However, results obtained with the indirect probes may be influenced by a number of factors such as changes in membrane lipid composition or interaction (particularly with the fluorescent probes) with reactive molecular species. In addition it is often difficult to know if the observed responses of these probes represent changes across the plasma membrane or changes within intracellular compartments. In an attempt to minimize these limitations we used two lipophilic probes with different molecular structures, the fluorescent cyanine dye di-O-C_5(3) and the radiolabeled molecule $TPMP^+$. Qualitatively similar data were obtained with both probes in normal neutrophils and the response could not be related to direct effects of superoxide ion or reactive species. The similarity in responses suggests that the first phase (decreased fluorescence and decreased accumulated $TPMP^+$) may reflect in part at least a depolarization. Based on ion substitution studies, both di-O-C_5(3) and $TPMP^+$ appear to provide a method for monitoring potential changes in neutrophils (28, 30, 49).

The data in this paper provide evidence that neutrophils obtained from patients with CGD have an abnormality of the plasma membrane compatible with defective stimulus induced alteration of membrane potential in response to f-Met-Leu-Phe and PMA. This conclusion is further supported by the recent finding of Lehrer et al. that in neutrophils from patients with CGD potassium and rubidium fluxes stimulated by phagocytic stimuli are abnormal (31). In the current paper the abnormality in neutrophils from patients with CGD could not be related to an abnormal resting membrane potential. Furthermore, CGD PMN hyperpolarized normally in response to valinomycin, implying the resting PMN have normal potassium gradients. The minimal defect of CGD cells to A23187

(compared to the PMA and f-Met-Leu-Phe abnormalities) suggests that the defect in CGD PMN is not related to abnormal calcium gradients in the resting cell. Since A23187 appears to elicit calcium-triggered potassium efflux in cultured macrophages (57), defect in CGD cells may be related to abnormal calcium-stimulated potassium fluxes. The membrane abnormality of CGD does not appear to result from an abnormal resting state of CGD PMN, but rather a defect related specifically to activation.

The observations made in neutrophils from patients with CGD, together with the studies using PMN obtained from other patients with defective neutrophil chemotaxis (Chediak-Higashi syndrome and the syndrome of elevated IgE, recurrent pyogenic infection, and abnormal chemotaxis [Job's syndrome] [36, 37]), suggests the defect is unique to patients with CGD. However studies of cells from patients with other forms of neutrophil dysfunction, such as myeloperoxidase deficiency and glucose-6-phosphate dehydrogenase deficiency, are still needed to substantiate this conclusion.

The defect observed with di-O-C_5(3) and $TPMP^+$ in neutrophils from patients with CGD, which clearly have locomotory capacity and degranulate their lysosomal enzymes upon stimualtion with phagocytic or soluble particles, suggests that alteration of membrane potential is more closely related to activation of oxygen metabolism than to stimulation of chemotaxis or degranulation. However, the abnormal rate of degranulation and the subtle defect of chemotaxis (55, 58–62; Table I and Fig. 8) in neutrophils from patients with CGD suggest membrane potential may modulate these latter functions. Alternatively, superoxide or other species that are absent in PMN from patients with CGD may regulate degranulation and chemotaxis. Delineation of these possibilities and clarification of the significance of the apparent disorder of neutrophil activation in the pathophysiology of chronic granulomatous disease will await additional study.

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Note added in proof: While this manuscript was in press another study was published that also indicated abnormal response in chronic granulomatosus disease polymorphonuclear leukocytes stimulated with phorbol myrisate acetate as monitored with the membrane potential sensitive probe, di-S-C3-(5). Whitin, J. C., C. E. Chapman, E. R. Simons, M. E. Chovaneic, and H. J. Cohen. 1980. *J. Biol. Chem.* **255**: 1874–1878.

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