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Research Article

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Functional Maturation of Membrane Potential Changes and Superoxide-producing Capacity during Differentiation of Human Granulocytes

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Abstract. The alterations of stimulus-induced membrane potential changes, superoxide (O_2^-)-producing capacity and phagocytic activity during differentiation of human granulocytes were investigated in the human leukemia cell lines HL-60 and KG-1 differentiating in vitro and in human leukemic granulocytes obtained from chronic myelogenous leukemia patients. HL-60 cells incubated with dimethyl sulfoxide or with retinoic acid showed progressively increasing O_2^- production as well as membrane potential changes (depolarization) on contact with phorbol myristate acetate or the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine, with a concomitant increase in the proportion of mature cells of the granulocytic type. Phagocytosis of latex particles, yeast, and oil droplets appeared 24 h after incubation with dimethyl sulfoxide and anteceded the increment of O_2^- production and membrane potential changes, both of which appeared concomitantly 3 d after incubation with dimethyl sulfoxide. Similar findings were observed when immature and mature granulocytes obtained from chronic myelogenous leukemia patients were stimulated by phorbol ester, the chemotactic peptide, or calcium ionophore A23187, and the amount of O_2^- production was parallel to the magnitude of membrane potential changes. HL-60 and KG-1 cells incubated for 1–6 d with phorbol myristate acetate showed neither O_2^- production nor membrane potential changes on contact with phorbol ester, chemotactic peptide, or A23187, although such cells resembled macrophages morphologically, and their phagocytic activity was significantly increased. O_2^- production and membrane potential changes in normal granulocytes induced by phorbol ester, chemotactic peptide and A23187 were inhibited by 2-deoxyglucose. These findings indicate that the O_2^--producing system and the system provoking membrane potential changes may develop concomitantly as human granulocytes mature and differentiate, and that the development of these systems and of phagocytic activity may be independently regulated.

Introduction

Exposure of human granulocytes to a variety of soluble stimuli results in transmembrane potential changes and activation of oxidative metabolism, during which oxygen is converted to superoxide (O_2^-), hydrogen peroxide, and hydroxyl radical (1–4). Membrane potential changes are proposed to be closely associated with activation of oxidative metabolism. This proposal is based on two distinct findings: (*a*) Membrane potential changes antecede O_2^- production (2); and (*b*) membrane potential changes are markedly reduced or entirely absent in granulocytes obtained from patients with chronic granulomatous disease (CGD)[1] but normal in granulocytes from patients with other granulocytic disorders such as Chediak-Higashi syndrome and hereditary myeloperoxidase deficiency (3, 4). On the other hand, it has

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1. *Abbreviations used in this paper:* CGD, chronic granulomatous disease; di-O-C_5(3), 3,3′-dipentyloxacarbocyanine; di-S-C_3(5), 3,3′-dipropylthiodicarbocyanine; DMSO, dimethylsulfoxide; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; HBSS, Hanks' balanced salt solution; KRP, Krebs-Ringer's phosphate buffer; PMA, phorbol myristate acetate.

been recently reported that membrane potential changes are unlikely to be involved in the triggering process that leads to activation of the oxidative metabolism in bovine granulocytes stimulated by phorbol myristate acetate (PMA), since the rate and extent of depolarization elicited by PMA are reduced in a low Na^+ medium, whereas activation of granulocyte respiration by PMA is not impaired in these situations (5). An alternative approach to answer the question whether membrane potential changes are necessary or responsible for the activation of human granulocytes involves studies of the alteration of membrane potential changes during differentiation of human granulocytes, since many functions of human granulocytes, including O_2^- production and phagocytosis, appear as the cells mature and differentiate. In this paper, we investigated the alterations of stimulus-induced membrane potential changes, O_2^- producing capacity and phagocytic activity during differentiation of human granulocytes, using human leukemia cell lines, HL-60 and KG-1 cells, differentiated in vitro by dimethyl sulfoxide (DMSO), retinoic acid or PMA (6–9), and human leukemic granulocytes obtained from chronic myelogenous leukemia patients. The results presented here show that human granulocytes gain the ability to exhibit membrane potential changes in response to PMA, *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), and calcium ionophore A23187 as the cells mature and differentiate.

Methods

Chemicals. Cytochrome *C* type VI, superoxide dismutase, retinoic acid, PMA, FMLP, and gramicidin were purchased from Sigma Chemical Co., St. Louis, MO; 2-deoxyglucose from Wako Chemical Co., Osaka, Japan; di-S-C_3(5) (3,3′-dipropylthiodicarbocyanine) and di-O-C_5(3) (3,3′-dipentyloxacarbocyanine) were provided from Japanese Research Institute for Photosensitizing Dyes, Okayama; and calcium ionophore A23187 was a gift from Dr. R. J. Hosley, Eli Lilly International Corp., Indianapolis, IN.

PMA, FMLP, A23187, and gramicidin were dissolved in DMSO and diluted with Hanks' balanced salt solution (HBSS) (Nissui Seiyaku Co., Tokyo) immediately before use. The final concentration of DMSO was <0.25%. Retinoic acid was dissolved in methanol. Stock solutions of di-S-C_3(5) and di-O-C_5(3) were made in ethanol and kept in the dark at 4°C.

Cell culture. HL-60 cells (a generous gift from Dr. R. C. Gallo, National Institutes of Health, Bethesda, MD) and U-937 cells (a generous gift from Dr. K. Sagawa, Kurume University, Fukuoka, Japan) were grown in RPMI-1640 medium (Nissui Seiyaku Co.) supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). For induction of differentiation of HL-60 cells, cells were seeded at 2×10^5 cells/ml and grown in the presence or absence of 1.3% DMSO, 1 μM retinoic acid, or 4 nM PMA. KG-1 cells (a generous gift from Dr. D. W. Golde, University of California, Los Angeles, School of Medicine) were grown in alpha medium (Flow Laboratories, Mclean, VA) supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). For induction of differentiation of KG-1 cells, cells were seeded at 2×10^5 cells/ml and grown in the presence or absence of 16.2 nM PMA. Cell viability was determined by the erythrosine B dye exclusion test.

For morphological assessment of the cells, Cytospin slide preparations were prepared using a Schandon Cytospin centrifuge (Schandon Southern Instruments, Inc., Sewickley, PA) and stained with Wright-Giemsa. Differential cell counting was performed under a light microscope on 200–400 stained cells for each experimental point.

Preparation of cells. Cultured cells were harvested after 1–6 d of cultivation with or without inducers of differentiation, washed three times, and suspended in HBSS, Krebs-Ringer's phosphate buffer (KRP, pH 7.4), or RPMI-1640 medium according to the experiments.

Normal granulocytes from healthy adult donors were prepared as described (10), using dextran sedimentation and centrifugation with Conray-Ficoll (Conray from Mallinckrodt Inc., St. Louis, MO and Ficoll from Pharmacia Fine Chemicals, Inc., Piscataway, NJ). Contaminated erythrocytes in granulocyte fractions were removed by hypotonic lysis. Granulocyte fractions were suspended in HBSS or KRP and contained >97% granulocytes.

Granulocytes at various stages of maturation were prepared from three patients with chronic myelogenous leukemia, using dextran sedimentation and Conray-Ficoll. Immature and mature granulocytes were separated into three fractions. Fraction I was obtained from the interface layer, fraction II from the Conray-Ficoll layer, and fraction III from the bottom. The main constituents of fraction I were myeloblasts, promyelocytes, and myelocytes; those of fraction II were myelocytes, metamyelocytes, and band-form neutrophils; and those of fraction III were segmented neutrophils. Cells were suspended in HBSS, KRP, or RPMI-1640 medium.

Determination of O_2^- production. O_2^- was assayed spectrophotometrically by the reduction of ferricytochrome *C*, and the continuous assay was performed in a Hitachi 557 spectrophotometer (a double wavelength spectrophotometer; Hitachi Ltd., Tokyo), equipped with thermostatted cuvette holder as described (10). The cell suspension in HBSS was added to a 1-ml cuvette containing 66 μM ferricytochrome *C* to obtain final volume of 0.995–0.9975 ml. Final cell concentration was 1×10^6 cells/ml. The reaction mixture in a cuvette was preincubated at 37°C for 5 min. The cuvette was put in a thermostatted cuvette holder (37°C) of a spectrophotometer and the reduction of cytochrome *C* was measured at 550 nm with a reference wavelength at 540 nm. Various stimulating agents (2.5–5 μl) were added to the reaction mixture in cuvettes to obtain final volume of 1 ml and the desired concentrations of these agents, while the time course of cytochrome *C* reduction (the absorbance change at 550–540 nm) was followed on the recorder. The final concentrations of stimuli were 20 ng/ml PMA, 1 μM FMLP, and 1 μM A23187. Although FMLP-induced O_2^- production by human granulocytes is markedly enhanced in the presence of cytochalasin B (11), cytochalasin B was not used throughout the present experiments. Cytochrome *C* reduction by cells stimulated by these surface active agents was completely abolished by superoxide dismutase (40 μg/ml), and suggested to be specific for O_2^-. The production of O_2^- was calculated from cytochrome *C* reduced for 3 min after the addition of FMLP or A23187, and from the linear portion of cytochrome *C* reduction for PMA (12). The values of cytochrome *C* reduced in the resting states were subtracted from those in the stimulated states.

Determination of membrane potential changes. Changes in the transmembrane potential were measured by using the fluorescent carbocyanine dyes, di-S-C_3(5) and di-O-C_5(3) (3, 4). The fluorescence was measured with a Hitachi MPF-4 fluorescence spectrophotometer (Hitachi Ltd.), equipped with thermostatted cuvette holder (37°C). The cell suspension in HBSS was added to a 3-ml cuvette containing 1.57 μM di-S-C_3(5) or 0.25 μM di-O-C_5(3) to obtain a final volume of 3 ml. The concentration of ethanol in the reaction mixture was <0.08%, and this amount of ethanol had no effect on cell viability or fluorescence. Final cell con-

centration was 1×10^6 cells/ml. The cells were equilibrated with dyes for 10 min at 37°C before stimuli were added. The cell suspension was maintained by means of a magnetic flea and stirrer. The excitation and emission wavelength were set at 622 nm and 665 nm for di-S-C_3(5), and 460 nm and 510 nm for di-O-C_5(3), respectively. In these experiments, cell viability by the erythrosine B dye exclusion test was >98%.

Effect of 2-deoxyglucose on membrane potential changes and O_2^- production. For the experiments with 2-deoxyglucose, normal granulocytes were suspended in phosphate-buffered saline (Nissui Seiyaku Co.) containing 0.5 mM $CaCl_2$ and no glucose. The cells were preincubated with indicated concentrations of 2-deoxyglucose for 10 min at 37°C before PMA, FMLP, A23187, or gramicidin was added. The same concentrations of glucose were added to the controls.

Phagocytosis of latex particles, yeast, and oil droplets. Latex particles (Dow Chemical Co., Indianapolis, IN; 1.09 μm in diam) were added to the cell suspensions in RPMI-1640 medium and left in contact with the cells for 60 min at 37°C. Final concentrations were 1×10^{10} latex particles/ml and $0.5–1 \times 10^6$ cells/ml, respectively. Cells ingesting more than five latex particles were considered as cells capable of phagocytosis and at least 200 viable cells were examined in any experimental groups.

Yeast particles (*Saccharomyces cerevisiae;* Sigma Chemical Co.) were added to the cell suspensions in HBSS containing 5% fresh human serum and left in contact with the cells for 30 min at 37°C. Final concentrations were 6.25×10^7 yeast particles/ml and 2.5×10^6 cells/ml, respectively. After centrifugation, the cell pellets were stained with Ziehl's carbolfuchsin solution. 1 vol of 11% fuchsin solution in ethanol and 10 vol of 5% phenol in distilled water were mixed, and diluted 10-fold with HBSS to obtain a working solution of Ziehl's carbolfuchsin solution. The yeast particles outside the cells were stained red, and the yeast particles completely ingested by the cells were protected from taking up the stain. The cells containing unstained yeast particles were considered as cells capable of phagocytosis.

A sonicated oil emulsion of 1,2,3,6-tetrahydrophthalic acid diisodecyl ester (Tokyo Kasei Co., Tokyo) containing oil red O dye (Schmid GMBH & Co., Stuttgart-Untertürkheim, Federal Republic of Germany) and *Escherichia coli* lipopolysaccharide (Difco Laboratories, Detroit, MI) was opsonized by incubation in fresh human serum for 30 min at 37°C, then incubated with cells (5×10^6 cells/ml) for 10 min at 37°C in KRP containing 5 mM glucose with or without 1 mM *N*-ethylmaleimide (13). Oil uptake was calculated from the absorbance at 515 nm of dioxane extracts of washed cell pellets and the previously determined absorbance at 515 nm of the emulsion. To determine the specific ingestion rate of oil droplets, the values obtained in the presence of *N*-ethylmaleimide were subtracted from those obtained in the absence of *N*-ethylmaleimide.

Results

Studies in normal granulocytes. Human granulocytes release O_2^- in response to various soluble stimuli such as PMA, FMLP, and A23187, which appear to stimulate human granulocytes through different mechanisms as previously described (14). The representative time courses of O_2^- production induced by PMA, FMLP, and A23187 are shown in Fig. 1 (upper). These soluble stimuli also induced the fluorescence changes of di-S-C_3(5) and di-O-C_5(3), although the patterns of the fluorescence changes were different according to the stimuli and dyes used (Fig. 1). An increase and a decrease in fluorescence of di-S-C_3(5) may reflect an apparent depolarization and hyperpolarization, respectively, whereas, on the contrary, a decrease and an increase in fluorescence of di-O-C_5(3) may reflect an apparent depolarization and hyperpolarization, respectively (3, 4). The fluorescence changes of di-S-C_3(5) induced by PMA, FMLP, and A23187 were similar to one another, and showed a biphasic response with an increase (apparent depolarization) followed by a decrease (apparent repolarization or hyperpolarization) in fluorescence (Fig. 1, middle). On the other hand, the fluorescence

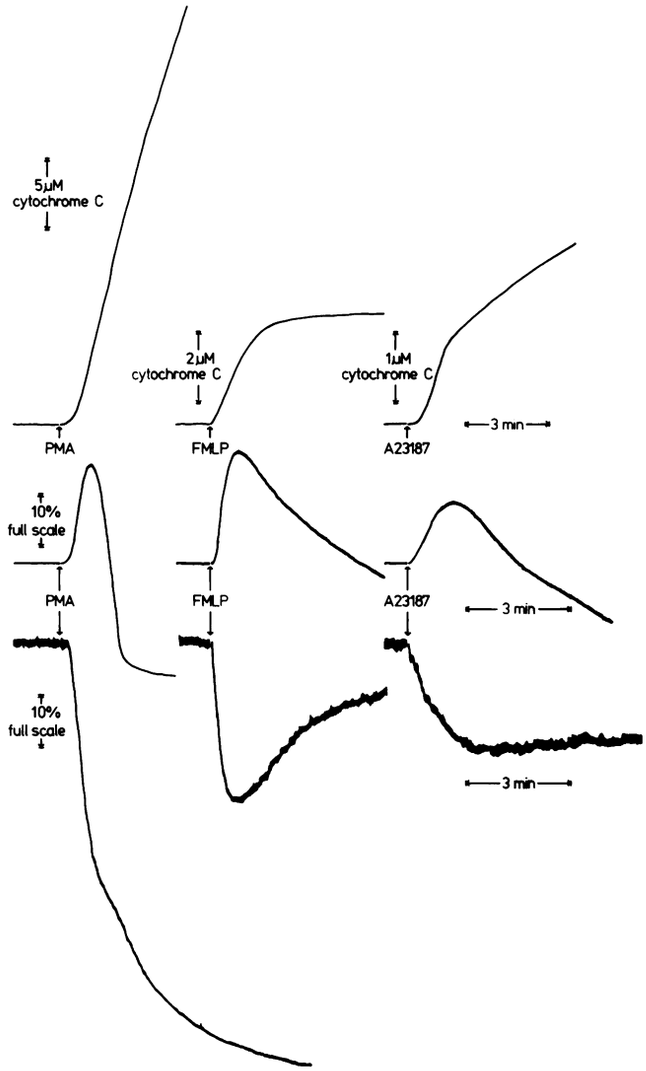

Figure 1. O_2^- production and membrane potential changes in normal granulocytes. Final cell concentration was 1×10^6 cells/ml. (*Upper*) Time courses of O_2^- production induced by PMA (20 ng/ml), FMLP (1 μM), and A23187 (1 μM). (*Middle*) Time courses of the fluorescence changes of di-S-C_3(5) induced by PMA (20 ng/ml), FMLP (1 μM), and A23187 (1 μM). (*Lower*) Time courses of the fluorescence changes of di-O-C_5(3) induced by PMA (20 ng/ml), FMLP (1 μM), and A23187 (1 μM).

changes of di-O-C_5(3) induced by PMA and A23187 showed a monophasic response with a sustained decrease in fluorescence (apparent depolarization), whereas that induced by FMLP showed a biphasic response with a decrease (apparent depolarization) followed by an increase in fluorescence (apparent repolarization) (Fig. 1, lower). The first phase of the response (apparent depolarization) was consistently observed regardless of the stimuli and dyes used, whereas the second phase of the response was different according to the stimuli and dyes used. These differences may be partly explained by the susceptibility of di-S-C_3(5) for oxidation by myeloperoxidase-H_2O_2-halide system and the varying potency of different stimuli to induce secretion of myeloperoxidase and H_2O_2. It has been reported that di-S-C_3(5), but not di-O-C_5(3), is easily oxidized by myeloperoxidase-H_2O_2-halide system and loses its fluorescence (15). Then, a decrease in fluorescence of di-S-C_3(5) (apparent hyperpolarization) may be an artifact resulting from the oxidation of di-S-C_3(5) by myeloperoxidase-H_2O_2-halide system. If this is the case, a decrease in fluorescence of di-S-C_3(5) could be used as an indicator of secretion of myeloperoxidase and H_2O_2 (15).

Studies in mature and immature granulocytes obtained from chronic myelogenous leukemia patients. To study the alterations of membrane potential changes, O_2^- production, and phagocytosis during differentiation of human granulocytes, peripheral blood granulocytes from chronic myelogenous leukemia patients were separated into three fractions according to the stages of differentiation. As shown in Figs. 2 and 3, the amount of O_2^- production induced by PMA, FMLP, or A23187 increased as the cells matured and differentiated. The magnitude of membrane potential changes induced by PMA, FMLP, or A23187 also increased as the cells matured when either di-S-C_3(5) or di-O-C_5(3) was used as a probe, and the amount of O_2^- production was parallel to the magnitude of membrane potential changes (Figs. 2 and 3). The time courses of O_2^- production and membrane potential changes (the fluorescence changes of di-S-C_3[5] and di-O-C_5[3]) in these mature and immature granulocytes were similar to those in normal granulocytes. The phagocytosis of latex particles, yeast, and oil droplets was also increased as the cells matured and differentiated (Fig. 3). The activity of phagocytosis was parallel to the amount of O_2^- production and the magnitude of membrane potential changes.

Studies in HL-60 cells differentiated by DMSO, retinoic acid, or PMA. The results shown in Figs. 2 and 3 indicate that stimulus-induced membrane potential changes, as well as O_2^- production and phagocytosis, may develop during differentiation of cells into mature granulocytes. To obtain additional evidence, we studied the alterations of membrane potential changes, O_2^- production, and phagocytosis during differentiation of HL-60 cells, a human promyelocytic leukemia cell line. It has been established that HL-60 cells differentiate into more mature cells of the granulocytic type when the cells are incubated with 1.3% DMSO or 1 μM retinoic acid (6, 7), and we also confirmed these findings as previously described (16). As shown in Figs. 4 and 5, HL-60 cells incubated with 1.3% DMSO for 3 d showed significant and remarkable O_2^- production on contact with PMA or FMLP,

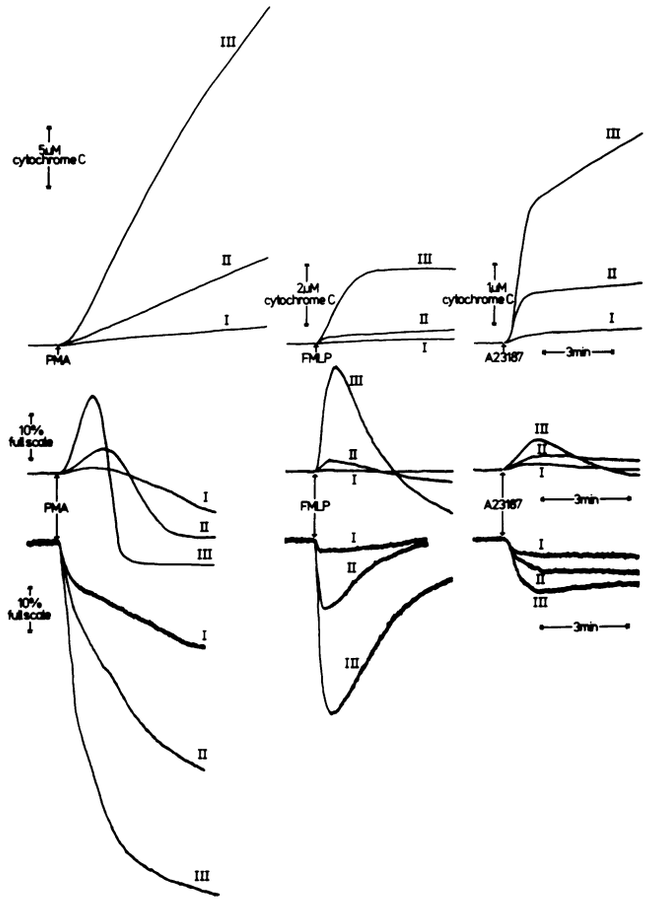

Figure 2. O_2^- production and membrane potential changes in mature and immature granulocytes obtained from chronic myelogenous leukemia patients. Granulocytes were separated into three fractions according to the stages of maturation. The main constituents of fraction I were myeloblasts, promyelocytes, and myelocytes; those of fraction II were myelocytes, metamyelocytes, and band-form neutrophils; and those of fraction III were segmented neutrophils. Final cell concentration was 1×10^6 cells/ml. (*Upper*) Time courses of O_2^- production induced by PMA (20 ng/ml), FMLP (1 μM), and A23187 (1 μM). (*Middle*) Time courses of the fluorescence changes of di-S-C_3(5) induced by PMA (20 ng/ml), FMLP (1 μM), and A23187 (1 μM). (*Lower*) Time courses of the fluorescence changes of di-O-C_5(3) induced by PMA (20 ng/ml), FMLP (1 μM), and A23187 (1 μM).

as compared with cells incubated without DMSO, with a concomitant increase in the proportion of mature cells (16, 17). The amount of O_2^- production further increased and approached that of normal granulocytes when HL-60 cells were incubated for 6 d with DMSO. The time courses of O_2^- production in mature HL-60 cells stimulated by PMA or FMLP were similar to those in normal granulocytes. The membrane potential changes (apparent depolarization) were also observed when HL-60 cells incubated with DMSO for 3 d were challenged with PMA or FMLP, and the magnitude of membrane potential

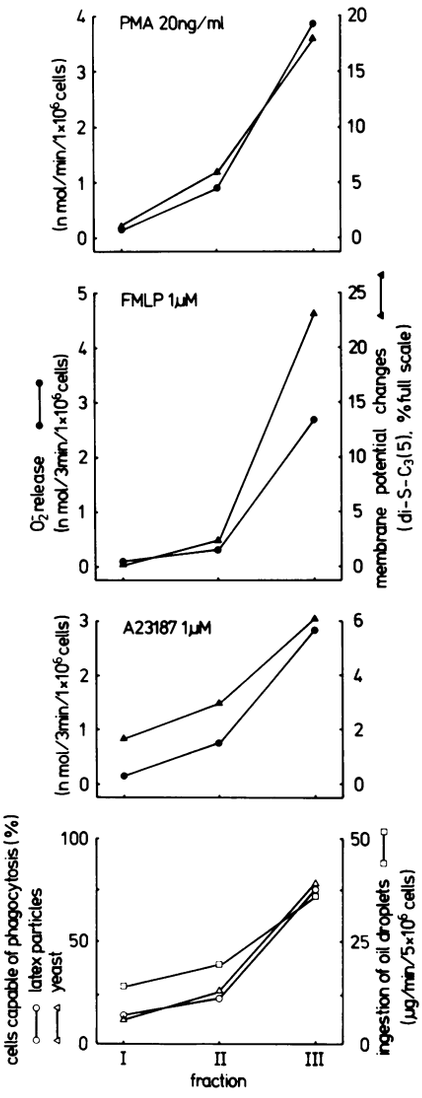

Figure 3. O_2^- production, membrane potential changes and phagocytic activity in mature and immature granulocytes obtained from chronic myelogenous leukemia patients. The calculated values of the data shown in Fig. 2 were plotted in upper three panels. The values of membrane potential changes are expressed as the maximum fluorescence changes (an increase of fluorescence) of di-S-C_3(5) induced by PMA (20 ng/ml), FMLP (1 μM), and A23187 (1 μM). ●, O_2^- production; ▲, membrane potential changes. (*Lower*) Phagocytic activity of cells. Phagocytic activity is expressed as the percentage of cells ingesting (○) latex and (△) yeast particles, and as (□) the ingestion rate of oil droplets. The main constituents of each fraction were the same as described in the legend of Fig. 2.

changes further increased when the cells were incubated with DMSO for 6 d. The amount of O_2^- production was parallel to the magnitude of membrane potential changes (Figs. 4 and 5). In contrast to normal granulocytes, which showed a biphasic response on contact with PMA when di-S-C_3(5) was used as a probe (Fig. 1), mature HL-60 cells showed a monophasic response on contact with PMA, a sustained increase in fluorescence (Fig. 4). This sustained increase continued for at least 10 min. On the other hand, FMLP-induced membrane potential changes in mature HL-60 cells were similar to those in normal granulocytes. The phagocytosis of latex particles, yeast, and oil droplets was also increased when HL-60 cells were incubated with 1.3% DMSO for 1–6 d (Fig. 6) (17). The significant increment of phagocytic activity appeared 24 h after incubation with DMSO and anteceded the significant increment of O_2^- production and membrane potential changes, both of which appeared concomitantly 3 d after incubation with DMSO.

As shown in Fig. 5 (lower), HL-60 cells incubated with 1 μM retinoic acid for 2 d also showed significant O_2^- production as well as membrane potential changes on contact with PMA, as compared with cells incubated without retinoic acid. The amount of O_2^- production and the magnitude of membrane potential changes further increased when HL-60 cells were incubated for 4 d with retinoic acid, and the amount of O_2^- production was parallel to the magnitude of membrane potential changes (Fig. 5). The time courses of PMA-induced O_2^- pro-

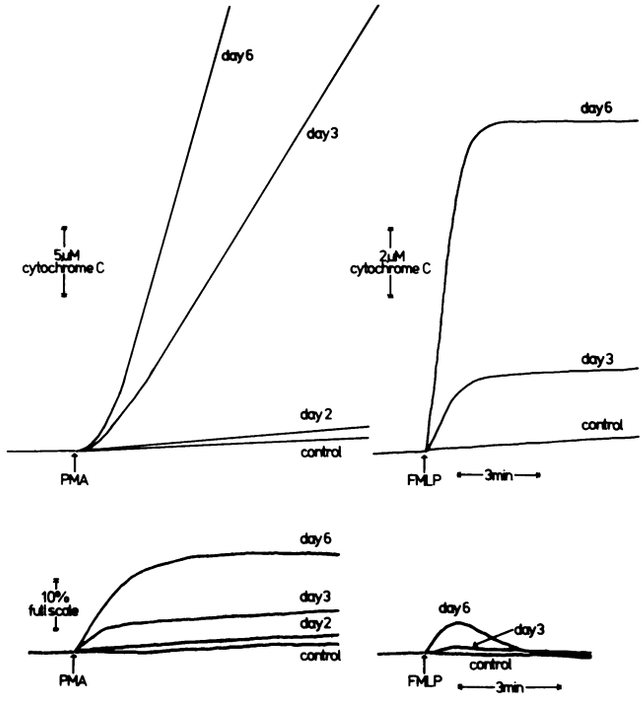

Figure 4. O_2^- production and membrane potential changes in HL-60 cells differentiated by DMSO. Cells were harvested at 2, 3, and 6 d after cultivation with or without DMSO. Final cell concentration was 1×10^6 cells/ml. (*Upper*) Time courses of O_2^- production induced by PMA (20 ng/ml) and FMLP (1 μM). (*Lower*) Time courses of the fluorescence changes of di-S-C_3(5) induced by PMA (20 ng/ml) and FMLP (1 μM).

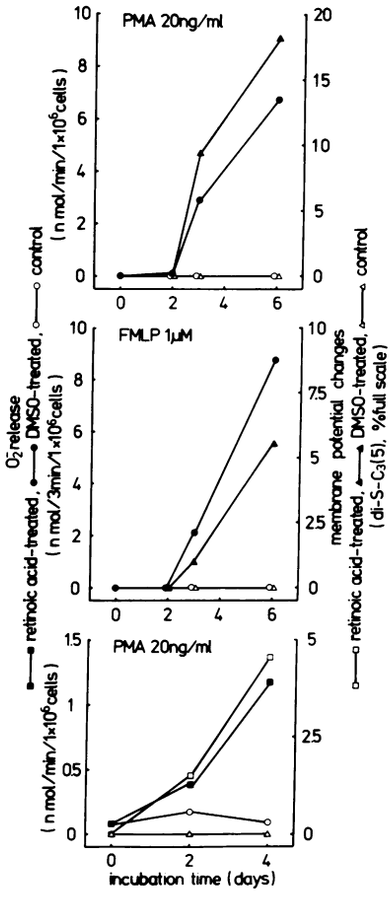

Figure 5. O_2^- production and membrane potential changes in HL-60 cells differentiated by DMSO and retinoic acid. The calculated values of the data shown in Fig. 4 were plotted in upper and middle panels. PMA-induced O_2^- production and membrane potential changes in HL-60 cells differentiated by retinoic acid are shown in lower panel. The values of membrane potential changes are expressed as the maximum fluorescence changes of di-S-C_3(5) induced by PMA (20 ng/ml) and FMLP (1 μM). ○ and △, O_2^- production and membrane potential changes in control cells, respectively. ● and ▲, O_2^- production and membrane potential changes in HL-60 cells differentiated by DMSO, respectively. ■ and □, O_2^- production and membrane potential changes in HL-60 cells differentiated by retinoic acid, respectively.

duction in mature HL-60 cells differentiated by retinoic acid were similar to those in normal granulocytes. However, the time courses of PMA-induced membrane potential changes in mature HL-60 cells differentiated by retinoic acid were different from those in normal granulocytes and similar to those in mature HL-60 cells differentiated by DMSO (data not shown). In contrast to mature HL-60 cells differentiated by DMSO, mature HL-60 cells differentiated by retinoic acid showed neither O_2^- production nor membrane potential changes on contact with FMLP. The lack of appearance of receptors for FMLP on mature HL-60 cells differentiated by retinoic acid (18) may be responsible for the lack of response to FMLP. The phagocytosis of latex particles was also increased when HL-60 cells were incubated with retinoic acid for 1–6 d (Fig. 6).

On the other hand, HL-60 cells incubated with PMA for 1–6 d showed neither O_2^- production nor membrane potential changes on contact with PMA, FMLP, or A23187 (data not shown), although such cells resembled macrophages morphologically (8) and the phagocytosis of latex particles, yeast, and oil droplets was significantly increased (Fig. 6).

Studies in KG-1 and U-937 cells. We studied membrane potential changes, O_2^- production, and phagocytosis by KG-1 cells, a human myelogenous leukemia cell line, and by U-937 cells, a human monocyte cell line (9, 19). KG-1 cells incubated with PMA for 1–6 d differentiated into macrophagelike cells morphologically (9), and the phagocytosis of latex particles, yeast, and oil droplets was significantly increased (Fig. 6). However, these mature KG-1 cells showed neither O_2^- production nor membrane potential changes on contact with PMA or FMLP. In addition, ~10% of U-937 cells could phagocytose latex particles, and these cells showed neither O_2^- production nor membrane potential changes on contact with PMA or FMLP (data not shown).

Effect of 2-deoxyglucose on membrane potential changes and O_2^- production in normal granulocytes. To further clarify whether membrane potential changes may result from the simple membrane perturbation or the energy-dependent metabolism triggered by the interaction between the plasma membrane and the stimuli, we studied the effects of 2-deoxyglucose, an inhibitor of glycolysis, on membrane potential changes and O_2^- production induced by PMA, FMLP, and A23187. As shown in Table I, 2-deoxyglucose inhibited membrane potential changes as well as O_2^- production induced by all these stimuli in a dose-dependent fashion, although membrane potential changes were less sensitive to the inhibitory effect of 2-deoxyglucose than O_2^- production. The resting membrane potential (the resting level of fluorescence intensity) was not affected by preincubation with 2-deoxyglucose (20 mM) for at least 20 min at 37°C.

Effect of A23187 and gramicidin on HL-60 cells and normal granulocytes. It has been recently reported that membrane potential changes induced by PMA and FMLP are markedly reduced or entirely absent in CGD granulocytes, whereas calcium ionophore A23187 induces an essentially normal membrane potential changes in CGD granulocytes (4). The minimal defect of CGD granulocytes to A23187 has been proposed to imply that the resting CGD granulocytes have normal calcium gradients (4). However, the results shown in Fig. 2 and Table I suggest that A23187-induced membrane potential changes are characteristic for mature granulocytes and require energy from glycolysis. To further clarify whether A23187-induced membrane potential changes simply reflect the calcium gradients in the cells, we compared the effects of A23187 with those of sodium ionophore gramicidin. As shown in Figs. 1 and 7, calcium ionophore A23187 induced membrane potential changes and O_2^- production in normal granulocytes, but not in undifferentiated

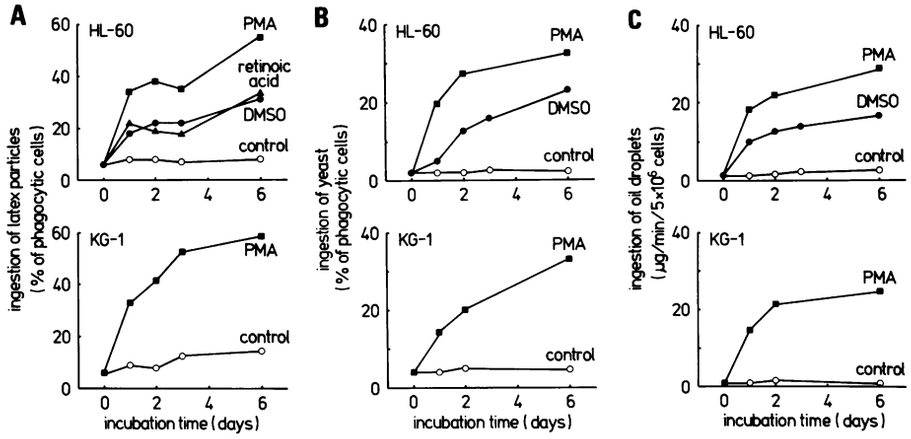

Figure 6. Phagocytic activity of differentiated HL-60 and KG-1 cells. (*Upper*) Phagocytic activity of HL-60 cells differentiated by DMSO (●), retinoic acid (▲), or PMA (■). (*Lower*) Phagocytic activity of KG-1 cells differentiated by PMA (■). ○, Phagocytic activity of control cells. Phagocytic activity is expressed as the percentage of cells ingesting (*A*) latex and (*B*) yeast particles, and as (*C*) the ingestion rate of oil droplets.

HL-60 cells. On the other hand, sodium ionophore gramicidin induced membrane potential changes (depolarization) in undifferentiated HL-60 cells as well as normal granulocytes (Fig. 7), without inducing O_2^- production in both types of cells (data not shown). Furthermore, in contrast to A23187, gramicidin-induced membrane potential changes were not inhibited by 2-deoxyglucose (Table I).

Discussion

Several lipophilic probes are available for the determination of membrane potential of cells such as granulocytes, which are too small for the insertion of a microelectrode. These probes include the lipophilic cations such as triphenylmethylphosphonium and tetraphenylphosphonium, and the carbocyanine dyes such as di-S-C_3(5) and di-O-C_5(3) (2–5). The use of these different probes has provided evidence that human granulocytes show rapid membrane potential changes in response to various stimuli. The patterns of membrane potential changes obtained by using di-O-C_5(3) were identical to those obtained by using triphenylmethylphosphonium or tetraphenylphosphonium; that is, PMA and A23187 induced a sustained depolarization, and FMLP induced a transient depolarization followed by a partial repolarization (Fig. 1) (4, 5). On the other hand, when di-S-C_3(5) is used as a probe, the first phase of the response can be used as an indicator of membrane depolarization and the second phase can be used as an indicator of secretion of myeloperoxidase and H_2O_2 (15), although the interpretation of the second phase is complicated.

Table I. Effect of 2-Deoxyglucose on Membrane Potential Changes and O_2^- Production in Normal Granulocytes

Stimuli	2-Deoxyglucose	Membrane potential changes	O_2^- production
	mM	*% control*	*% control*
PMA	2	78.1±6.5	34.2±4.4
	20	47.7±2.6	22.2±0.1
FMLP	2	63.8±2.6	11.4±2.9
	20	43.4±4.7	7.5±0.6
A23187	2	74.1±2.0	37.4±5.4
	20	51.9±3.1	8.4±1.1
Gramicidin	2	103.4±4.5	ND
	20	103.6±0.5	ND

Cells were suspended in phosphate-buffered saline containing 0.5 mM $CaCl_2$. The cells were preincubated with indicated concentrations of 2-deoxyglucose for 10 min at 37°C before PMA (20 ng/ml), FMLP (1 μM), A23187 (1 μM), or gramicidin (0.5 μg/ml) was added. The same concentrations of glucose were added to the controls. Membrane potential changes were measured by the fluorescence changes of di-O-C_5(3), and the values of maximum fluorescence changes were compared. Two or three experiments were averaged and each experiment was done in triplicate. ND, not done.

The fluorescence changes of di-S-C_3(5) in mature HL-60 cells stimulated by PMA were different from those in normal granulocytes or mature leukemic granulocytes obtained from chronic myelogenous leukemia patients, and rather similar to those in myeloperoxidase-deficient granulocytes reported by Whitin et al. (15). It has been demonstrated that the content of myeloperoxidase in HL-60 cells markedly diminishes when the cells are differentiated by DMSO (20), and we also observed these findings (data not shown). The marked diminution of myeloperoxidase activity and/or the marked reduction of myeloperoxidase secretion in mature HL-60 cells may partly explain the sustained increase of fluorescence of di-S-C_3(5) induced by PMA, since mature HL-60 cells released significant amount of O_2^- in response to PMA, and then H_2O_2 by dismutation of O_2^-. Another explanation is that the sustained increase of fluorescence of di-S-C_3(5) induced by PMA may reflect the defective maturation of the plasma membrane in HL-60 cells. However, this possibility seems to be unlikely, since the fluorescence changes of di-S-C_3(5) in mature HL-60 cells stimulated by FMLP were similar to those in normal granulocytes. It appears that membrane potential changes induced by FMLP may be char-

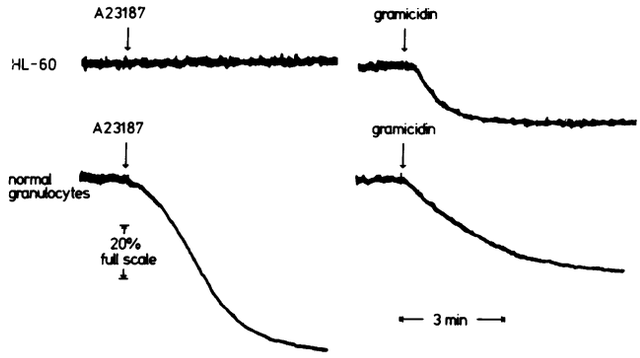

Figure 7. Membrane potential changes in HL-60 cells and normal granulocytes induced by A23187 and gramicidin. Cells were suspended in phosphate-buffered saline containing 0.5 mM $CaCl_2$. Final cell concentration was 5×10^5 cells/ml. Membrane potential changes were measured by the fluorescence changes of di-O-C_5(3). HL-60 cells (*upper*) and normal granulocytes (*lower*) were equilibrated with di-O-C_5(3) for 10 min at 37°C before A23187 (1 μM) or gramicidin (0.5 μg/ml) was added.

acterized by an initial depolarization followed by a repolarization, and those induced by PMA and A23187 may be characterized by a sustained depolarization.

Many types of secretory cells show membrane potential changes upon activation of secretion (21). Although it is plausible that membrane potential changes might alter membrane properties and activate the metabolism of cells, there is at present no convincing evidence for these possibilities. In fact, it is possible that membrane potential changes might reflect simple membrane perturbation provoked by the binding of stimulus to its receptors and might not be involved in the process of activating cell metabolism. If membrane potential changes might be necessary or responsible for the activation of human granulocytes, it is expected that membrane potential changes would appear as the cells mature and differentiate. In the present experiments, we used two different systems: (*a*) peripheral blood granulocytes obtained from chronic myelogenous leukemia patients, and (*b*) HL-60 cells differentiated in vitro by DMSO and retinoic acid. The results show that human granulocytes gain the ability to exhibit membrane potential changes in response to PMA, FMLP, and A23187 as the cells mature and differentiate. These results highly strengthen the hypothesis that stimulus-induced membrane potential changes may play an important role in stimulus-response coupling of human granulocytes, and do not reflect simple membrane perturbation. Another evidence supporting possible participation of membrane potential changes in stimulus-response coupling may come from the experiments with 2-deoxyglucose. 2-deoxyglucose inhibited membrane potential changes as well as O_2^- production stimulated by PMA, FMLP, and A23187, indicating that (*a*) membrane potential changes induced by these stimuli may be the active metabolic events requiring energy from glycolysis, or (*b*) membrane potential changes themselves may be the passive events resulting from the preceding energy-dependent processes.

The lack of membrane potential changes in undifferentiated HL-60 and KG-1 cells in response to PMA does not imply that these cells have no receptors for PMA, since both cells, when incubated with PMA, differentiated into macrophagelike cells and specific receptors for PMA have been demonstrated in HL-60 cells (22). These findings indicate that the binding of stimulus to its receptors is not sufficient in itself to provoke membrane potential changes, and that the certain system is necessary for provoking membrane potential changes in response to surface stimulation. The present results show that this system may develop during differentiation of human granulocytes. The results also suggest that PMA-induced commitment of differentiation of HL-60 and KG-1 cells into macrophagelike cells is not preceded by the rapid membrane potential changes.

The significant increment of phagocytic activity in HL-60 cells appeared 24 h after incubation with DMSO and anteceded the significant increment of O_2^- production and membrane potential changes, both of which appeared concomitantly 3 d after incubation with DMSO (Figs. 4–6). In addition, HL-60 and KG-1 cells, when incubated with PMA, differentiated into macrophagelike cells and their phagocytic activity was markedly increased (Fig. 6). However, these macrophagelike cells showed neither O_2^- production nor membrane potential changes in response to PMA, FMLP, or A23187. Thus, it appears that development of O_2^--producing capacity and the system provoking membrane potential changes may be closely correlated to each other, and development of these two functions may be dissociated with development of phagocytic activity. The lack of O_2^- production and membrane potential changes in HL-60 and KG-1 cells differentiated by PMA does not imply that normal monocytes/macrophages do not show these responses, since it has been demonstrated that these cells also show O_2^- production and membrane potential changes in response to appropriate stimuli (23) and we also found that human peripheral blood monocytes showed O_2^- production (12) as well as membrane potential changes (data not shown) in response to PMA. One possibility is that HL-60 and KG-1 cells differentiated by PMA may be functionally deficient (24). However, it has been recently reported that differentiation of human monocytes into macrophages in vitro is accompanied by an apparent reduction in the capacity to produce O_2^- and H_2O_2 (25). Therefore, another possibility is that HL-60 and KG-1 cells differentiated by PMA may show certain stages of differentiation, and these cells are not necessarily functionally deficient.

It is unlikely that calcium ionophore A23187-induced membrane potential changes simply reflect the calcium gradients in the cells, since (*a*) A23187 did not induce membrane potential changes in undifferentiated HL-60 cells, (*b*) the magnitude of membrane potential changes induced by A23187 increased as the cells matured and differentiated, and (*c*) A23187-induced membrane potential changes were inhibited by 2-deoxyglucose. In these respects, membrane potential changes induced by A23187 were similar to those induced by PMA and FMLP. In marked contrast to A23187, the sodium ionophore gramicidin induced adequate membrane potential changes in undifferen-

tiated HL-60 cells as well as normal granulocytes without inducing O_2^- production. These findings suggest that membrane potential changes by themselves are not sufficient to trigger the activation of oxidative metabolism. Another important difference from the effects of A23187 is that gramicidin-induced membrane potential changes were not inhibited by 2-deoxyglucose. It is likely that gramicidin depolarizes the plasma membrane directly without requiring energy. These results may indicate that energy-dependent membrane potential changes may be coupled with activation of the oxidative metabolism.

The O_2^--producing system is proposed to consist of several components: NADPH oxidase, flavin, and cytochrome *b* (26, 27). Roberts et al. (28) have recently reported that HL-60 cells differentiated by DMSO acquire cytochrome *b* and the increase in the concentrations of cytochrome *b* parallels the increase in PMA-stimulated oxidative activity. These findings and the present results taken together suggest that the O_2^--producing system and the system provoking membrane potential changes may develop concomitantly as human granulocytes mature and differentiate, and that development of these systems and phagocytic activity may be independently regulated. It is likely that deficient development of these systems may be responsible for deficient functions of CGD granulocytes.

CGD granulocytes fail to show O_2^- production as well as membrane potential changes in response to various stimuli except A23187 (3, 4). A23187 induces essentially normal membrane potential changes in CGD granulocytes without inducing O_2^- production (4). It is possible that A23187 may bypass a certain process that is deficient in CGD granulocytes to induce membrane potential changes. Further investigations into the mechanisms of membrane potential changes may help to shed light on the pathophysiology in CGD granulocytes and to elucidate the role of membrane potential changes in stimulus-response coupling.

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