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Recombinant Granulocyte Colony-Stimulating Factor and Lipopolysaccharide Maintain the Phenotype of and Superoxide Anion Generation by Neutrophils

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Superoxide anion (O_2^-) generation by human blood neutrophils induced by phorbol myristate acetate, formyl-methionyl-leucyl-phenylalanine, and monoclonal antibody YI51 was measured 24 h after incubation in medium alone, medium with recombinant human granulocyte colony-stimulating factor (rG-CSF), and medium with lipopolysaccharide (LPS). Monoclonal antibody YI51 was able to bind to neutrophils and induce O_2^- generation after the addition of anti-mouse immunoglobulin antibody as a cross-linking agent. In the 24-h culture, there was no significant difference in neutrophil survival among the three cultures. The amount of O_2^- generated by neutrophils in control medium markedly decreased compared with that before culture. However, cells in medium with rG-CSF or LPS maintained the ability to generate O_2^- well or moderately, respectively. Thus, the activity maintained by rG-CSF and LPS was neutralized by the anti-G-CSF serum. Furthermore, significant amounts of G-CSF were detected in supernatants of neutrophils cultured with LPS for 24 h. It was not detectable, however, in control supernatants. To examine whether the phenotype of the plasma membrane of cells changed in the 24-h culture, expression of CD16 (FcR III) and YI51 antigens was analyzed by flow cytometry. The expression of CD16 and YI51 antigens on cells cultured with rG-CSF or LPS was maintained compared with that of control cells. These observations thus indicate that G-CSF is one of the factors essential to maintain the functioning and phenotype of mature neutrophils.

Two human colony-stimulating factors (CSFs) with granulocyte-macrophage activity have been identified from several sources of crude-condition medium based on differences in hydrophobicity. CSFα stimulates the functional activity of neutrophils, eosinophils, and macrophages, as well as the proliferation of those progenitor cells. The effect of CSFβ is, however, restricted to neutrophils and their progenitor cells (13, 15, 21). By recombinant DNA technology, recombinant human granulocyte-macrophage CSF (rGM-CSF) and recombinant human granulocyte CSF (rG-CSF) are produced. They are analogous to CSFα and CSFβ, respectively, in their activities (15).

With respect to the effect of rGM-CSF on mature neutrophils, several recent studies have shown that neutrophils respond to rGM-CSF with decreased migratory activity (7), enhanced phagocytosis, antibody-dependent cellular cytotoxicity (14), and superoxide anion (O_2^-) production in response to chemoattractant formyl-methionyl-leucyl-phenylalanine (FMLP) (22). At present, the functional effects of rG-CSF on mature neutrophils remain, on the whole, unclear.

Gram-negative bacterial endotoxin, lipopolysaccharide (LPS), has diverse biological effects on neutrophils. For example, LPS has been shown to prime human neutrophils for an enhanced respiratory burst and production of leukotriene B4 in response to a second stimulus such as phorbol myristate acetate (PMA) or FMLP (3, 8). However, to our knowledge, these effects of LPS have been observed in only short-term cultures, i.e., within 2 h. The effect of LPS in long-term cultures and the mechanism of action by which it exerts its biological effects on the cells are unknown.

In this work, we found that rG-CSF was able to maintain plasma membrane structure and O_2^- generation in mature neutrophils in a 24-h culture. In addition, LPS was shown to have a similar effect on neutrophils. This effect of LPS could be explained by the autorelease of G-CSF from neutrophils cultured with LPS.

MATERIALS AND METHODS

Reagents. Cytochalasin E, superoxide dismutase (type 1; from bovine blood), ferricytochrome *c* (type IV; from horse heart), and FMLP were purchased from Sigma Chemical Co., St. Louis, Mo. PMA was purchased from P-L Biochemicals, Inc., Milwaukee, Wis. LPS (*Escherichia coli*, type B5) was purchased from Difco Laboratories, Detroit, Mich. Phosphate-buffered saline was purchased from Nissui Pharmaceutical Co., Ltd., Tokyo, Japan. RPMI 1640 medium and fetal bovine serum were purchased from GIBCO Laboratories, Grand Island, N.Y. Anti-Leu-IIb (CD16) antibody was purchased from Becton Dickinson Immunocytometry Systems, Mountain View, Calif. $F(ab')_2$ fragments of goat anti-mouse immunoglobulin antibody were purchased from Cooper Biomedical, Inc., Malvern, Pa. rG-CSF (5×10^7 U/mg of protein as determined by stem cell colony assay) and rabbit anti-G-CSF serum were gifts of Chugai Pharmaceutical Co., Ltd., Tokyo, Japan. Recombinant human tumor necrosis factor (rTNF) (3×10^6 U/mg of protein as determined by cytotoxicity assay with L929 cells) was the gift of Dainippon Pharmaceutical Co., Ltd., Tokyo, Japan. Recombinant human interleukin-1 (rIL-1) (5×10^7 U/mg of protein as determined by growth inhibition assay with A375

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S1 cells) was a gift from Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan. All media and reagents at various concentrations used in this study were endotoxin free (detection limit, 0.1 ng/ml) as determined by *Limulus* amoebocyte lysate assay (Wako Chemical Co., Osaka, Japan).

Preparation and culture of human peripheral blood neutrophils. Heparinized peripheral blood was obtained from healthy donors. Neutrophils were prepared by a one-step density gradient centrifugation method, using Mono-Poly Resolving Medium (Flow Laboratories, Inc., McLean, Va.), as described by Ferrante and Thong (5). The neutrophil-rich layer formed by centrifugation at 300 × *g* for 30 min was harvested, and the cells were centrifuged at 250 × *g* for 10 min, followed by three washings in phosphate-buffered saline. The purity of these preparations was determined by preparing cytocentrifuge smears and staining with Difquick (Difco). In all experiments, neutrophils thus prepared were >94% pure and >99% viable as determined by trypan blue exclusion. Contamination of monocytes was <0.5% as determined by nonspecific esterase staining. Cells were incubated in suspension at a concentration of 3×10^6 in 3 ml of RPMI 1640 supplemented with 10% fetal bovine serum containing various concentrations of LPS, rG-CSF, rTNF, and rIL-1. All cultures were incubated at 37°C in a fully humidified atmosphere of 5% CO_2 in air.

Determination of amount of O_2^- released. O_2^- determination was performed as described previously (11). Briefly, 1 ml of a reaction mixture containing 100 μM cytochrome *c* and 5×10^5 cells in HEPES buffer (17 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, pH 7.3) was preincubated in a plastic cuvette for spectrophotometry at 37°C for 10 min. Thereafter, PMA (1.6×10^{-7} M) or FMLP (10^{-5} M) and cytochalasin E (5 μg/ml) were added to the reaction mixture, and the rate of superoxide dismutase-inhibitable reduction of cytochrome *c* was measured continuously by recording the A_{550} to A_{540} increase (molar absorption coefficient, 1.9×10^4) with a Hitachi 556 two-beam spectrophotometer. To prevent sedimentation of the cells, a cell mixture was attached to the cuvette. Monoclonal antibody (MAb) YI51 is able to bind to neutrophils and induce O_2^- generation after the addition of anti-mouse immunoglobulin antibody as a cross-linking agent (9). To assay O_2^- generation by neutrophils stimulated by MAb YI51, 5×10^5 cells were incubated in 800 μl of HEPES buffer and 200 μl of culture supernatant from the cloned hybridoma cells (YI51) for 1 h at 4°C. The cells were washed twice with phosphate-buffered saline to remove the free antibody and then suspended in 1 ml of HEPES buffer. O_2^- generation by the cells was assayed after the addition of $F(ab')_2$ fragments of goat anti-mouse immunoglobulin antibody (5 μg/ml) and cytochalasin E (5 μg/ml).

Assay of LDH. Lactate dehydrogenase (LDH) released from cells into the culture medium was assayed by using an LDH-S · RI kit (Kokusai Shiyaku, Tokyo, Japan) and a TBA-60S spectrophotometer (Toshiba, Tokyo, Japan).

Assay of G-CSF. Supernatants of neutrophils (10^6/ml) cultured with medium alone or LPS (1.0 μg/ml) for 24 h were collected and assayed to determine the amount of G-CSF released, as described by Motojima et al. (16). Briefly, duplicate 0.2-ml samples of standards or supernatants were dispensed into anti-G-CSF antibody-coated tubes followed by 0.5 ml of 0.05 M Tris hydrochloride buffer (pH 8.0) containing 0.25% bovine serum albumin, 0.05% Tween 20, 2% polyethylene glycol, 0.9% NaCl, and 0.1% NaN_3. After the tubes were incubated for 2 h at room temperature, horseradish peroxidase-labeled $F(ab')_2$ fragments of rabbit anti-G-CSF antibody (0.1 ml) were added and the tubes were incubated for a further 2 h. The tubes were washed three times with 2 mM Tris hydrochloride buffer (pH 8.0), and 1 ml of substrate solution was added to each tube. After 1 h of incubation at room temperature in the dark, the reaction was stopped with 1 ml of 4 N H_2SO_4 and the A_{492} values were read. The detection range of G-CSF in this assay was 30 to 2,000 pg/ml.

Binding of MAb YI51 and anti-CD16 antibody to cells. Cells, 10^6, before and after culture were washed three times in phosphate-buffered saline and incubated in 0.5 ml of MAb YI51 or 5 μl of anti-CD16 antibody at 4°C for 60 min. The cells were then washed three times in phosphate-buffered saline and incubated with fluorescein isothiocyanate-conjugated $F(ab')_2$ fragments of goat anti-mouse immunoglobulin antibody (Organon Teknika, Malvern, Pa.) at 4°C for 30 min. Stained cells were washed and then analyzed on an EPICS C flow cytometer (EPICS Div., Coulter Electronics, Inc., Hialeah, Fla.).

Statistical analyses. The statistical significance of the differences between the test groups was analyzed by Student's (two-tailed) *t* test.

RESULTS

Survival of neutrophils. In the first set of experiments, the survival of peripheral blood neutrophils cultured with medium alone and medium with rG-CSF (1.0 ng/ml) or LPS (1.0 μg/ml) was examined (Fig. 1). At 12-h culture time, cell loss was not observed under the various culture conditions. Although the survival rate of the cells in cultures with G-CSF or LPS appeared to be higher than in the control culture at 24 h of culture, the difference was not statistically significant. After continued incubation, cell numbers continued to decline, and at 48 h the survival rate of the cells cultured with G-CSF or LPS was significantly different from that of cells cultured with medium alone ($P < 0.05$). When the LDH in culture supernatants thought to be released from dead cells was measured, the increase in level of LDH correlated with a decrease in cell survival (Fig. 1).

On light microscopy of cells cultured with medium alone for 24 h, 45 to 60% of cells showed nuclear pyknosis or chromatin condensation. In contrast, the same morphological features were seen in only 10 to 30% of cells cultured with rG-CSF and LPS. When cell size was compared, the lengths of the long axes of cells with and without morphological change were 11.2 ± 2.1 and 14.8 ± 1.2 μm, respectively. This was determined by measurement of 30 cells of each group.

O_2^- generation by neutrophils cultured with rG-CSF or LPS for 24 h. In the next set of experiments, the amounts of O_2^- generation by neutrophils cultured with medium containing rG-CSF (1.0 ng/ml) or LPS (1.0 μg/ml) for 24 h were compared (Table 1). In response to PMA, FMLP, and MAb YI51, cells cultured with control medium were capable of generating O_2^- release, but the amounts were significantly lower than those by cells cultured with rG-CSF or LPS. Cells cultured with rG-CSF showed greater response to all stimuli than cells cultured with medium alone or medium plus LPS. When compared with the amount of O_2^- generation by neutrophils before culture, cells in medium with rG-CSF and LPS maintained their abilities to generate O_2^- well and moderately, respectively.

The amount of O_2^- generation by neutrophils cultured for 24 h, in response to PMA, was observed to be concentration

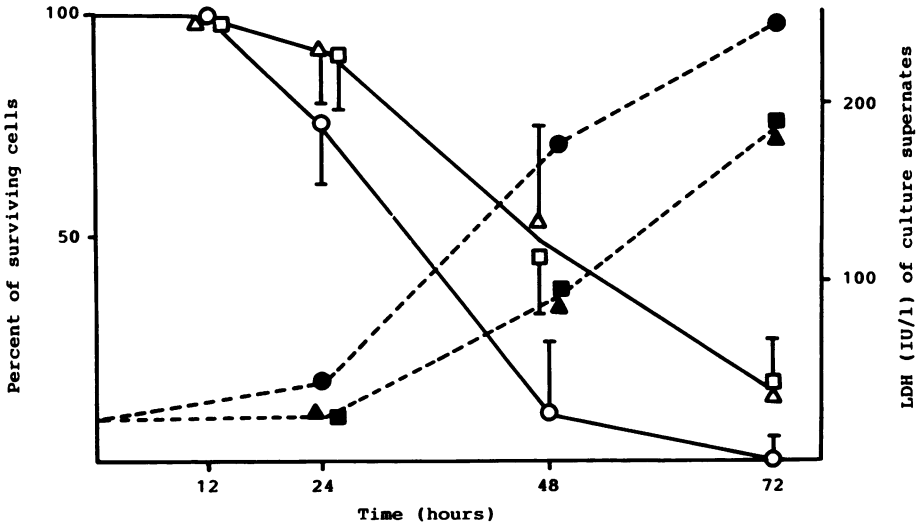

FIG. 1. Survival of peripheral blood neutrophils in culture and LDH levels of culture supernatants. Cells (3×10^6/3 ml per well) were placed in 12-well culture plates and cultured with control medium (○), rG-CSF at 1.0 ng/ml (△), or LPS at 1.0 μg/ml (□). The number of surviving cells determined by trypan blue exclusion was counted at various times. Data represent mean ± standard deviation of five separate experiments. LDH levels of culture supernatants of control (●), rG-CSF (1 ng/ml) (▲), or LPS (1.0 μg/ml) (■) were measured 0, 24, 48, and 72 h after the start of culture. The standard deviation at every point had a range of <20% of the mean (n = 3).

dependent in medium with rG-CSF or LPS (Fig. 2). When compared with the control, cells cultured with rG-CSF at 10 pg/ml were shown to have enhanced O_2^- generation, which reached a plateau at 100 pg of rG-CSF per ml. To ensure a maximal effect of LPS on enhanced generation of O_2^-, 10 to 100 ng of LPS per ml was required (three experiments). In contrast, rIL-1 or rTNF had no or little activity in enhancing O_2^- generation in the 24-h culture (Fig. 2).

Abolishment of effect of rG-CSF and LPS on O_2^- generation by anti-G-CSF serum. Neutrophils were cultured with rG-CSF or LPS in the presence of rabbit anti-G-CSF serum for two reasons. One was to examine whether the maintenance of O_2^- generation by neutrophils cultured with rG-CSF for 24 h was truly due to the presence of rG-CSF. The other was to determine whether the effect of LPS could possibly be ascribed to G-CSF released from cells cultured with LPS for 24 h. The maintained generation of O_2^- induced by rG-CSF or LPS was neutralized by anti-G-CSF serum in cultures (Table 2). Moreover, this effect of LPS (0.1 μg/ml) on O_2^- generation was completely abolished in the presence of polymyxin B (5 μg/ml), whereas that of rG-CSF was unaffected (data not shown).

Release of G-CSF from neutrophils cultured with LPS. To confirm the above finding, supernatants of neutrophils cultured with LPS (1.0 μg/ml) for 24 h were assayed for G-CSF in four individual experiments. Supernatants of cells cultured with LPS contained G-CSF at 164.3 ± 63 pg/ml (mean ± standard deviation), ranging from 77 to 238 pg/ml, while control culture supernatant did not have detectable levels of G-CSF. Furthermore, in supernatants of monocytes (10^3/ml)

TABLE 1. Effects of rG-CSF or LPS on O_2^- generation by neutrophils in long-term culture

Cell culture	O_2^- generation (nmol/min per 5×10^5 cells)[a] after stimulus with:		
	PMA	FMLP	MAb YI51
Before culture	3.02 ± 0.64	3.17 ± 0.76	3.66 ± 0.92
24-h culture[b]			
Control	1.04 ± 0.22	0.64 ± 0.22	0.40 ± 0.29
rG-CSF (1.0 ng/ml)	3.09 ± 0.40[c]	2.63 ± 0.27[c]	2.36 ± 0.64[c]
LPS (1.0 μg/ml)	2.29 ± 0.85[c]	1.96 ± 0.20[c]	1.43 ± 0.73[c]

[a] Amount of O_2^- generation by neutrophils was measured after addition of PMA (1.6×10^{-7} M) or FMLP (10^{-5} M) and cytochalasin E (5 μg/ml). In O_2^- generation induced by MAb YI51, cells were incubated MAb YI51 for 1 h at 4°C and the amount of O_2^- generation was measured after addition of anti-mouse immunoglobulin antibody (5 μg/ml) and cytochalasin E (5 μg/ml). Data represent means ± standard deviations obtained from four separate experiments.

[b] Neutrophils were cultured with medium control, G-CSF (1.0 ng/ml), or LPS (1.0 μg/ml) for 24 h at 37°C.

[c] $P < 0.05$, Significantly different from control.

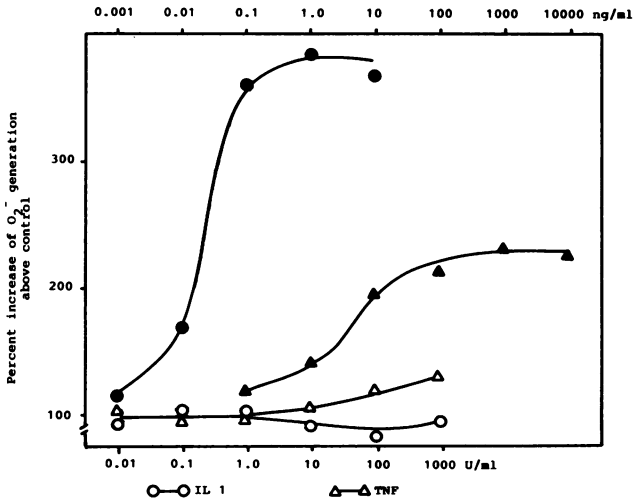

FIG. 2. Concentration-response effect of rG-CSF and LPS on O_2^- generation by neutrophils. Cells (3×10^6/3 ml per well) were placed in 12-well culture plates and cultured with control medium and various concentrations of rG-CSF (●), LPS (▲), rIL-1 (○), and TNF (△) for 24 h. Thereafter, the amount of O_2^- generated by cells was measured after addition of PMA (1.6×10^{-7} M) and compared with that of control neutrophils.

TABLE 2. Effect of anti-G-CSF antibody on O_2^- generation by neutrophils cultured with rG-CSF or LPS

Cell culture[a]	O_2^- generation (nmol/min per 5×10^5 cells)[b] after given stimulus	
	PMA (Ab−/Ab+)	MAb YI51 (Ab−/Ab+)
Control	1.3/1.15	0.65/0.65
rG-CSF (0.1 ng/ml)	3.26/1.05 (100)[c]	2.84/0.70 (98)
LPS (1.0 μg/ml)	2.74/1.28 (91)	2.10/0.91 (82)

[a] Neutrophils were cultured with medium alone, rG-CSF (0.1 ng/ml), and LPS (1.0 μg/ml) in the presence of a 1/1,000 dilution of rabbit anti-G-CSF serum or nonimmune rabbit serum for 24 h.

[b] O_2^- generation by neutrophils was measured as described in Table 1, footnote *a*. The data shown are representative of three experiments.

[c] Values in parentheses reflect the percentages of augmented O_2^- generation inhibited by anti-G-CSF antibody.

cultured with LPS for 24 h, G-CSF was not detectable (data not shown).

Expression of CD16 and YI51 antigens on plasma membrane of neutrophils. To investigate whether expression of CD16 and YI51 antigens on neutrophils changed in the 24-h culture, we used flow cytometry (Fig. 3). Both antigens are functional: CD16 is FcR III with a molecular weight of 50,000 to 70,000 (6), and YI51 antigen is involved in O_2^- generation (9). Cells before culture and at 3 h of culture formed a single spot defined by forward light scatter (cell size) and right-angle scatter (irregularity). More than 97% of those cells expressed CD16 and YI51 antigens. In the 24-h culture, small-sized neutrophils appeared and formed another spot. This was coincident with the finding observed by light microscopy. Comparison of the density of the original spot with that of the new spot showed that a greater number of cells changed in size in control medium than in the medium with rG-CSF or LPS. When expression of CD16 and YI51 antigens on neutrophils of original size was compared, control cells lost expression of both antigens to a greater extent than cells cultured with rG-CSF. In three individual experiments, YI51 and CD16 antigens were always detected on >90 and 80% of cells cultured with rG-CSF, respectively. In the cells cultured with medium alone, however, YI51 and CD16 antigens were detected on <70 and 60%, respectively. The expression of both antigens on cells cultured with LPS varied in the three individual experiments. Figure 3 shows the lowest and highest extents of these expressions.

Next, the expression of both antigens on small-sized neutrophils, which formed a new spot in flow cytometry, was analyzed (bottom panel of Fig. 3). When cells cultured even with rG-CSF for 24 h changed in size, those cells lost expression of YI51 and CD16 antigens on the plasma membrane to a greater extent than cells of the original size which were cultured with medium alone or medium with LPS or rG-CSF.

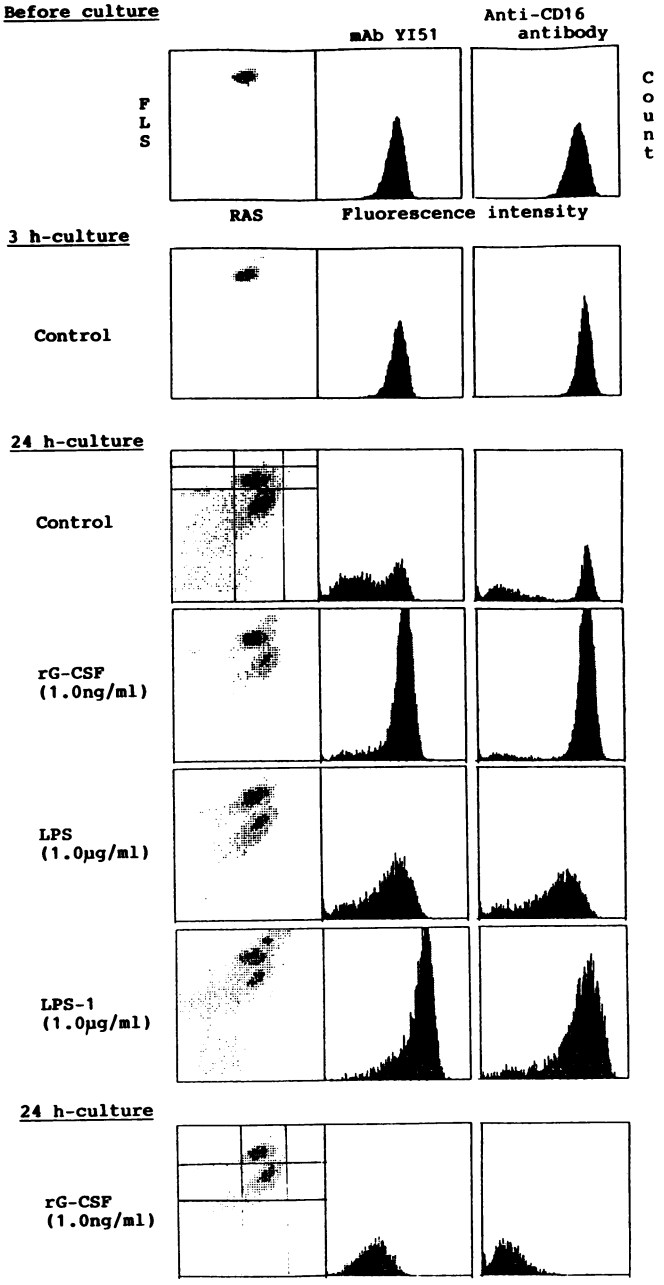

FIG. 3. Fluorescence intensity of neutrophils stained with MAb YI51 and anti-CD16 antibody. Cells (3×10^6/3 ml per well) were cultured with control medium, rG-CSF (1.0 ng/ml), or LPS (1.0 μg/ml) for up to 24 h and stained with MAb YI51 and anti-CD16 antibody. The left-hand panels are scatter profiles of forward light scatter (cell size) and right-angle scatter (irregularity) of individual cells. The middle and right-hand panels display histograms of fluorescence intensity (logarithmic scale) versus cell numbers. In profiles of flow cytometry of cells in 24-h culture, the upper four panels represent fluorescence intensity of cells of original size. The bottom panel represents fluorescence intensity of cells of smaller size.

DISCUSSION

Recently, rG-CSF and rGM-CSF have been proved capable of prolonging the survival of neutrophils and of neutrophils and eosinophils, respectively (1, 14). The present study confirmed the effect of rG-CSF on prolongation of survival of neutrophils. Neutrophils are relatively short-lived end-stage cells, considered to possess a mechanism of programmed cell death. Savill et al. (18) precisely reported the morphological changes in peripheral blood neutrophils cultured in vitro for up to 24 h. According to their report, neutrophils in culture are viable for up to 24 h, while about 50% of cells in 24-h culture show the morphological features of apoptosis. The latter is characterized by nuclear pyknosis or chromatin condensation together with cytoplasmic vacuolation on light microscopy. Our observations were the same

by light microscopy, measurement of LDH level in culture supernatants, and flow cytometry analysis. In the present study, we correlated the effect of rG-CSF on survival of neutrophils with structural change and function of aging neutrophils. rG-CSF was capable of delaying the time of programmed cell death or apoptosis of neutrophils in culture. At 24 h after the start of incubation, about 50% of the neutrophils cultured with medium alone showed morphological features of apoptosis, whereas only 10 to 30% of cells cultured with rG-CSF showed these features. In the analysis of flow cytometry of those cells, cells with apoptosis lost expression of YI51 and CD16 antigens to a greater extent than cells without apoptosis. This is a novel finding because cells with apoptosis were previously thought to have intact cell membranes, structually and functionally, regardless of changes in their nuclei (18, 24). Of greater interest is the fact that, in the analysis of cells considered to show original morphology without apoptosis, the culture with rG-CSF was capable of keeping expression of functional antigens such as YI51 and CD16 (FcR III) antigens on plasma membranes of the cells, whereas control cells began to lose such expression. These observations explain the maintaining effect of rG-CSF on O_2^- generation by neutrophils in response to MAb YI51. FMLP also induces O_2^- generation after binding to its receptor on the plasma membrane (4), while PMA is known to permeate cytoplasm and bind directly to protein kinase C. The latter is considered linked with activation of NADPH oxidase (23). In comparing the amounts of O_2^- generation of the control cells induced by PMA, FMLP, and MAb YI51, the response to PMA was relatively stable compared with that to FMLP and MAb YI51. When considered together, these findings suggest that the structure of the plasma membrane is affected to a greater extent than the cytoplasmic structure, both involved in O_2^- generation, in the process of apoptosis.

Berkow et al. (2) have reported that TNF is able to enhance O_2^- generation by neutrophils in response to FMLP or PMA after short-term incubation of neutrophils with TNF. In the present study, rTNF and IL-1, whose activities are similar to those of TNF (17), could not maintain O_2^- generation by neutrophils in 24-h culture, as opposed to G-CSF.

LPS is known to stimulate monocytes and neutrophils in various ways. LPS alone or LPS combined with gamma interferon stimulates monocytes to induce the release of IL-1, TNF, M-CSF, and G-CSF (10, 12). Neutrophils share a number of properties with monocytes, including their phagocytic activity, similar membrane receptors, a common progenitor cell, and generation of IL-1 (20). In the present study, we detected a significant amount of G-CSF released from neutrophils cultured with LPS. This autorelease of G-CSF could possibly explain the maintaining effect of LPS on O_2^- generation and survival. Sieff (19) has proposed the cascade of production of hematopoietic growth factors during infection with microorganisms; endotoxin-mediated release of IL-1 or TNF or both by monocytes greatly increases fibroblast and endothelial cell release of GM-CSF and G-CSF. The present study indicates another possible pathway of production of G-CSF in gram-negative bacterial infection.

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