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6-Formylpterin Intracellularly Generates Hydrogen Peroxide and Restores the Impaired Bactericidal Activity of Human Neutrophils

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The effects of 6-formylpterin on the impaired bactericidal activity of human neutrophils were examined *ex vivo*. When neutrophils isolated from fresh blood were incubated with 6-formylpterin, the intracellular production of hydrogen peroxide (H_2O_2) occurred. The H_2O_2 generation by 6-formylpterin in neutrophils occurred in the presence of diphenyleneiodonium (DPI), an inhibitor of NADPH-oxidase. When neutrophils were incubated with DPI, the killing rate of catalase-positive bacteria, *Escherichia coli* (*E. coli*) and *Staphylococcus aureus* (*S. aureus*), significantly decreased. This impaired bactericidal activity of the DPI-treated neutrophils was a mimic for chronic granulomatous disease (CGD). However, the killing rate of the DPI-treated neutrophils against *E. coli* and *S. aureus* significantly increased when 6-formylpterin was administered. Since 6-formylpterin intracellularly generates H_2O_2 independent from the NADPH-oxidase, it was considered to improve the impaired bactericidal activity of the DPI-treated neutrophils. The use of 6-formylpterin may serve as an option of therapy for CGD.

Key Words: **6-formylpterin; hydrogen peroxide; neutrophils; diphenyleneiodonium; chronic granulomatous disease; bactericidal activity.**

Neutrophils play a pivotal role in host defense through oxidative and nonoxidative mechanisms for killing invading microorganisms (1). Oxidative killing involves an NADPH-oxidase, which is assembled in the phagosomal membrane and converts oxygen to superoxide ($O_2^{\cdot -}$) when neutrophils ingest the microorganisms (2, 3). If the oxidative mechanism is impaired, immunodeficient diseases such as chronic granulomatous disease (CGD) occur (4). CGD is an inherited disorder characterized by recurrent life-threatening bacterial and fungal infection with granuloma formation, resulting from a defect in $O_2^{\cdot -}$ generation, which acts as a precursor of hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\cdot}OH$), and hypochlorous acid (HOCl), by stimulated neutrophils (5). Neutrophils of CGD normally phagocytize bacteria but are unable to kill certain organisms after ingestion. They can kill catalase-negative bacteria, such as *Diplococcus pneumoniae*, but they cannot kill catalase-positive bacteria, such as *E. coli* and *S. aureus*. Therefore, it is suggested that formation of H_2O_2 by bacteria within neutrophils can compensate for the defect in H_2O_2 production in CGD neutrophils, and that the H_2O_2 produced by bacteria, in concert with myeloperoxidase (MPO), account for the ability of the CGD neutrophils to kill catalase-negative bacteria but not to kill catalase-positive bacteria (6). In fact, it was shown that normal neutrophils and those from patients with CGD can cooperate to damage microorganisms by jointly owning the products of the oxidative burst (7). Therefore, it is suggested that neutrophils in CGD improve their bactericidal activity if available reactive oxygen species (ROS) exist around them, and among the ROS, H_2O_2 is most suitable for this aim due to its stability, permeability to cell membranes and cooperation with MPO. However, exogenously administered H_2O_2 is ineffective because it passes through the plasma membrane and will be instantly consumed in the cytosol (8). Otherwise, intracellularly generated H_2O_2 in neutrophils is desirable for this aim.

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6-Formylpterin is known as a potent XOD inhibitor with an inhibition constant (K_i) of approximately 0.6 nM (9, 10). Although 6-formylpterin does not usually occur *in vivo*, it is produced from folic acid *in vivo* in some pathological conditions, such as carcinoma (11). Recently, we showed that 6-formylpterin reacted with reducing agents in the cells, such as NADH, and intracellularly generated H_2O_2 which induced apoptosis in HL-60 cells, suppressed cell proliferation in PanC-1 cells and inhibited Fas-mediated apoptosis in Jurkat cells. The induction of apoptosis and the suppression of cell proliferation were observed when high concentrations of 6-formylpterin (greater than 1 mM) was applied to the cells and the inhibition of Fas-mediated apoptosis was observed when relatively low concentrations of 6-formylpterin (up to 500 μM) were applied (12). Although these biological responses were all observed in the transformed cell lines, the intracellular H_2O_2 generation by 6-formylpterin should also occur in nontransformed normal cells including neutrophils. Then, 6-formylpterin may be available as an intracellular H_2O_2 generator in neutrophils, and should improve the impaired bactericidal activity of CGD neutrophils.

In the present study, to test the above-mentioned hypothesis experimentally, we applied 6-formylpterin to human peripheral neutrophils *in vitro*. First, intracellular H_2O_2 production in normal neutrophils was confirmed. Then second, the effects of diphenyleneiodonium (DPI), an inhibitor of NADPH-oxidase (13), on the 6-formylpterin-induced intracellular H_2O_2 production were examined. Finally, it was determined if 6-formylpterin improves the impaired bactericidal activity of DPI-treated neutrophils, a good mimic for CGD (14).

MATERIALS AND METHODS

Reagents. 6-Formylpterin was synthesized in our laboratories and its purity was determined as previously described (15). Hanks' balanced salt solution (HBSS), fetal calf serum (FCS), and penicillin-streptomycin liquid were purchased from GIBCO (Grand Island, NY); 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was from Labotec Co. (Tokyo, Japan); and diethylenetriaminepentaacetic acid (DETAPAC) was from Nacalai Tesque (Kyoto, Japan). Amplex Red H_2O_2 assay kit and 2′,7′-dichlorofluorescin diacetate (DCFH-DA) were obtained from Molecular Probes (Eugene, OR). Other chemicals, such as diphenyleneiodonium (DPI) and zymosan, were purchased from Sigma Chemicals (St. Louis, MO).

Preparation of cells and opsonized zymosan. Human neutrophils were isolated from peripheral blood of healthy adult volunteers by sedimentation through two-step Percoll (Pharmacia, Uppsala, Sweden) gradients, as previously described (16). Freshly purified cells were cultured in HBSS and kept on ice until use. Zymosan was first activated by boiling, and then the activated zymosan was incubated and reacted with fresh AB human serum. The zymosan was resuspended in HBSS and used as opsonized zymosan (OZ).

EPR spectroscopy and spin trapping. To confirm if neutrophils incubated with 6-formylpterin or OZ produce ROS and to determine the original radicals, electron paramagnetic resonance (EPR) spectroscopy with a spin trap, DMPO, was used to directly measure oxygen radicals. The neutrophils were suspended in HBSS and stored on ice until use. The mixture of neutrophils (ca. 2×10^6), DETAPAC (0.08 mM), and 6-formylpterin (2 mM) or OZ (4 mg/ml) was incubated for 3 min at 37°C. In some cases, various agents, such as superoxide dismutase (SOD) and $FeSO_4$, were added to the mixture to determine the original radicals. Immediately after the addition of DMPO (36 mM), the mixture was transferred to a flat quartz EPR aqueous cell, which was fixed in the cavity of the EPR spectrometer. The EPR spectrum recording started exactly 40 s after adding DMPO. The EPR spectra were recorded on a spectrometer (Model JES-TE300, JEOL, Ltd., Tokyo, Japan). The EPR settings were as follows: microwave power: 5 mW; field: 335 ± 5 mT (9.4231 GHz); modulation: 0.079 mT; time constant: 0.03 s; amplitude: 1600; and sweep time: 1 min. The intensity of DMPO spin adducts and the hyperfine splitting constants (hfsc's) were calculated, based on the Mn^{2+} marker, which was inserted into the cavity of the EPR spectrometer.

H_2O_2 assay in neutrophils. Intracellular H_2O_2 generation in neutrophils was measured using DCFH-DA, an H_2O_2 sensitive dye (17). Neutrophils (1×10^7/ml in HBSS containing 2 μM DCFH-DA) were transferred to a quartz cuvette (4 ml) containing a magnetic stir bar, which was fixed in a spectrofluorimeter (F-2000, HITACHI, Tokyo, Japan), and the changes in the fluorescence of stirred cell suspensions were continuously monitored at an excitation wavelength of 488 nm, an emission wavelength of 530 nm, and a slit width of 10 nm. The cuvette was maintained at 37°C in a water-jacketed holder within the spectrofluorimeter. After allowing several min for the baseline measurements, 6-formylpterin (500 μM) or OZ (0.5 mg/ml) was added to the cuvette and the changes in fluorescence were measured.

Extracellular H_2O_2 release from neutrophils was measured using a highly sensitive and stable H_2O_2 probe, *N*-acetyl-3,7,-2-phenylethylamine dihydroxyphenoxazine (Amplex Red) (18). The fluorometric assay was conducted in a 96-well microplate as instructed by the manufacturer. The fluorescence was measured by a fluorometric microplate reader (Fluoroskan Ascent; Labsystems, Helsinki, Finland) with excitation and emission wavelengths of 544 and 590 nm, respectively. The fluorescence intensity was converted to the concentration of H_2O_2 using a standard curve.

Flow cytometry for detection of intracellular H_2O_2 in neutrophils. Flow cytometric analysis of intracellular H_2O_2 generation in neutrophils was performed using the probe DCFH-DA. The cells (1×10^6/ml) were preincubated with DPI (10 μM) in HBSS for 10 min at 37°C in a shaking bath, and then incubated with 2 μM DCFH-DA and various concentrations of 6-formylpterin for an additional 1 h, subsequently the cells washed and resuspend in phosphate-buffered saline (PBS). The amount of intracellular H_2O_2 was detected by the FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA) and analyzed using the CELLQuest software (Becton Dickinson).

Preparation of bacteria. E. coli NIHJ-JC2 and *S. aureus* ATCC12600, obtained from the American Type Culture Collection (Rockville, MD), were cultured in Trypto-Soya Broth (Nissui, Tokyo, Japan) at 37°C for 24 h in a shaking water bath. The bacteria were washed twice with PBS and suspended in HBSS. The concentration of bacteria was determined by the turbidity of the suspension using a densitometer (Photoelectric colorimeter, Klett Summerson Co., New York, NY) and was adjusted at 1×10^8 bacteria/ml.

Bactericidal assay. Bactericidal activity of neutrophils was determined by a standard technique (19). The reaction mixture contained 1×10^6 neutrophils, 2×10^6 *E. coli* or 5×10^5 *S. aureus*, 10% human AB serum, 0.1% gelatin, and HBSS in a total volume of 0.5 ml. After 10 min preincubation with or without 10 μM DPI, the tubes containing the reaction mixture were further incubated with or without 6-formylpterin at 37°C in a shaking water bath. Samples were removed at 60 min (*E. coli*) or 75 min (*S. aureus*), and suspended for

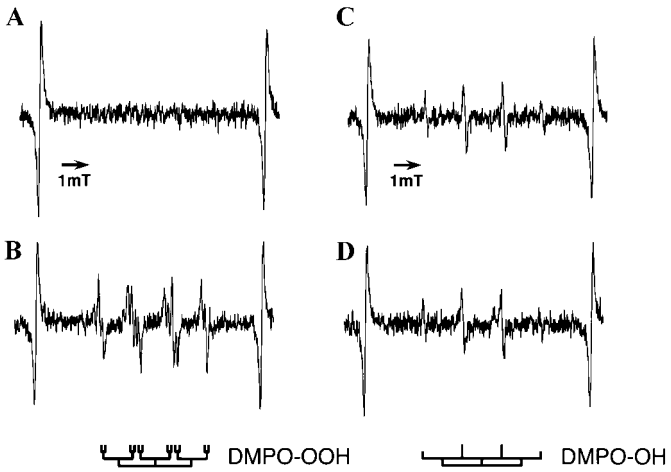

FIG. 1. EPR spectra obtained from neutrophils. The EPR spectra obtained from (A), the reaction mixture containing neutrophils (ca. 2×10^6) and 6-formylpterin (2 mM) and (B), the reaction mixture containing neutrophils and opsonized zymosan (OZ, 4 mg/ml). (C and D), similar to (A) and (B), respectively except that the reaction mixture containing $FeSO_4$, which induces Fenton reaction. Signals appearing both at the high and low field correspond to Mn^{2+} installed in the EPR cavity as a reference. The components, DMPO-OOH and DMPO-OH, are indicated by the stick diagram. Note that the DMPO-OOH signal is observed in only (B), while the DMPO-OH signal is observed in (C) and (D).

2 min in cold distilled water for disrupting neutrophils. An aliquot of suspension was plated on a pour plate made with Heart Infusion Agar (Nissui, Tokyo, Japan). After 24 h of incubation at 37°C, the colonies formed were counted.

Statistical analysis. Values were shown as means ± SD. Statistical comparisons were made using one-way analysis of variance, followed by Fisher protected least significant difference post hoc test to compare the group means.

RESULTS

Detection and Determination of the Original Radicals by EPR Spectroscopy

There were no oxygen radical signals in the EPR spectra obtained from the medium containing neutrophils and 6-formylpterin (Fig. 1, Trace A). However, OZ was added to the medium, prominent oxygen radical signals were observed (Fig. 1, Trace B), consisting of a doublet of a triplet with the hfsc's of a(N) = 1.41 mT, a(βH) = 1.14 mT and a(γH) = 0.11 mT, which is indicative of DMPO-OOH (20). These signals were totally quenched by SOD (data not shown), demonstrating that the signals were derived from trapping the superoxide anion radical ($O_2^{\cdot -}$). In turn, unlike OZ, 6-formylpterin did not yield $O_2^{\cdot -}$ in neutrophils. When iron sulfate ($FeSO_4$) was added to the medium containing neutrophils and 6-formylpterin, other oxygen radical signals were observed (Fig. 1, Trace C), consisting of a 1:2:2:1 quartet with the hfsc's of a(N) = 1.49 mT and a(βH) = 1.49 mT, which is indicative DMPO-OH (20), demonstrating that the signals were derived from trapping the hydroxyl radicals ($^{\cdot}OH$). These signals were also observed when $FeSO_4$ was added to the medium containing neutrophils and OZ (Fig. 1, Trace D). In the presence of ferrous ion (Fe^{2+}), $^{\cdot}OH$ is generated via the iron-catalyzed Fenton reaction from H_2O_2 (21). Therefore, it was revealed that H_2O_2 was produced in the medium containing neutrophils and 6-formylpterin as well as in the medium containing neutrophils and OZ.

H_2O_2 Generation in Neutrophils

The intracellular H_2O_2 generation in neutrophils was measured using the DCFH-DA probe. Although a spontaneous and gradual increase of fluorescence was observed from the start of the measurement, the administration of 6-formylpterin caused an apparent increase in fluorescence (Fig. 2A), demonstrating the intracellular H_2O_2 generation in the neutrophils. The administration of OZ also caused the increase in fluorescence. Recently, it was reported that the use of this probe to measure H_2O_2 production in cells is problematic, because the probe could be oxidized by any intra-

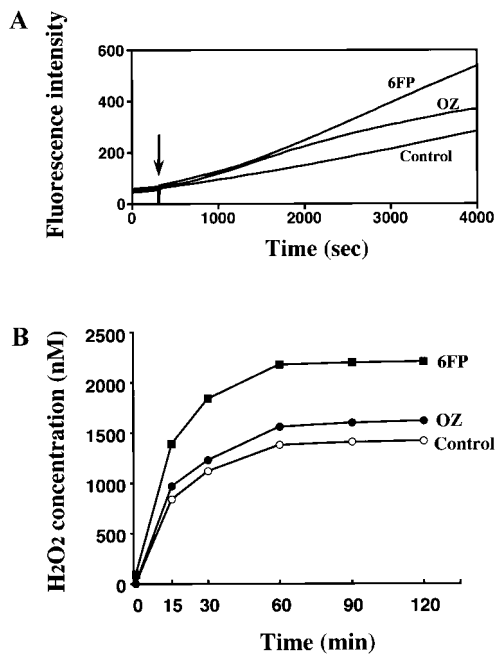

FIG. 2. H_2O_2 generation in neutrophils. (A) Intracellular H_2O_2 generation. Neutrophils were incubated with 2 μM DCFH-DA, an H_2O_2-sensitive dye, and the changes in fluorescent intensity were recorded continuously with a spectrofluorimeter. 6-Formylpterin (6FP, 500 μM) or opsonized zymosan (OZ, 0.5 mg/ml) was added to the neutrophils (ca. 1×10^7) in HBSS at the point indicated by an arrow. (B) Extracellular H_2O_2 release. Neutrophils (ca. 1×10^4) were incubated with 6FP (500 μM) or OZ (0.5 mg/ml) in HBSS containing Amplex Red, an H_2O_2-sensitive dye. Then, the changes in fluorescent intensity were measured intermittently with a fluorometric microplate reader. The H_2O_2 concentration was determined from the standard curve.

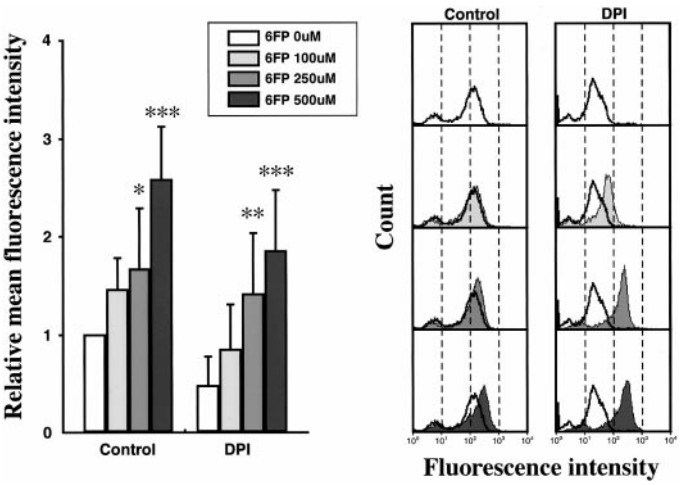

FIG. 3. Effects of DPI on intracellular H_2O_2 accumulation by 6-formylpterin. Neutrophils (1×10^6/ml) were preincubated with diphenyleneiodonium (DPI, 10 μM) for 10 min, and further incubated with DCFH-DA (2 μM) and 6-formylpterin (6FP, 0, 100, 250, and 500 μM). The relative mean fluorescence intensity was calculated when the mean fluorescence intensity obtained from the neutrophils incubated without DPI or 6-formylpterin was assigned to 1, and the values are shown as means ± SD ($n = 5$). Control, the neutrophils preincubated without DPI; DPI, the neutrophils preincubated with DPI. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$: significantly different from the values obtained from the neutrophils incubated without 6-formylpterin (6FP, 0 μM) in each group. The finding of a representative experiment is shown in the right half of the graph.

cellular oxidant, such as ferryl species, peroxynitrite, etc (22). However, in the present study, the H_2O_2 generation in neutrophils was already demonstrated in the preceding study using EPR. Therefore, the oxidation of DCFH-DA in this study is considered to be caused by H_2O_2.

The extracellular H_2O_2 release from neutrophils was measured using the probe Amplex Red. Although spontaneous H_2O_2 release from the medium containing neutrophils and Amplex Red was observed, the release apparently increased when 6-formylpterin was added to the medium (Fig. 2B), demonstrating the extracellular H_2O_2 release from neutrophils. The increase in the H_2O_2 release was also observed when OZ was added to the medium.

Effects of DPI on Intracellular H_2O_2 Generation by 6-Formylpterin

Flow cytometric analysis using the H_2O_2 sensitive probe DCFH-DA showed that the intracellular H_2O_2 generation by 6-formylpterin in neutrophils occurred even in the presence of DPI, an inhibitor of NADPH-oxidase (Fig. 3). The DCFH-DA fluorescence of the neutrophils incubated with 6-formylpterin increased in a dose-dependent manner (Fig. 3 Control). The fluorescence of the neutrophils incubated with DPI shifted to the left, compared with that of the neutrophils incubated without DPI. However, the fluorescence significantly increased with the increase of the concentrations of 6-formylpterin (Fig. 3 DPI). These results suggested that H_2O_2 generation by 6-formylpterin is independent of the NADPH-oxidase.

Effects of 6-Formylpterin on the Bactericidal Activity of Neutrophils

When the number of the colonies formed from the bacteria incubated without neutrophils was assigned to A, and that from the bacteria incubated with neutrophils, DPI and 6-formylpterin was assigned to B, the killing rate of the bacteria by the neutrophils was calculated as the percentage of (A–B) to A. According to this definition, the killing rate of untreated neutrophils against *E. coli* was 69 ± 13% (mean ± SD, $n = 10$). The killing rate of DPI-treated neutrophils significantly decreased to 27 ± 17% ($P < 0.001$ compared with untreated neutrophils). However, when DPI-treated neutrophils were incubated with 250 and 500 μM 6-formylpterin, the killing rates increased significantly to 44 ± 19% ($P < 0.05$ compared with DPI-treated neutrophils) and to 52 ± 17% ($P < 0.01$ compared with DPI-treated neutrophils), respectively (Fig. 4A). The killing rate of untreated neutrophils did not increase significantly using 6-formylpterin (data not shown).

Similarly, the killing rate of untreated neutrophils against *S. aureus* was calculated to be 51 ± 6% (mean ± SD, $n = 5$). The killing rate of DPI-treated

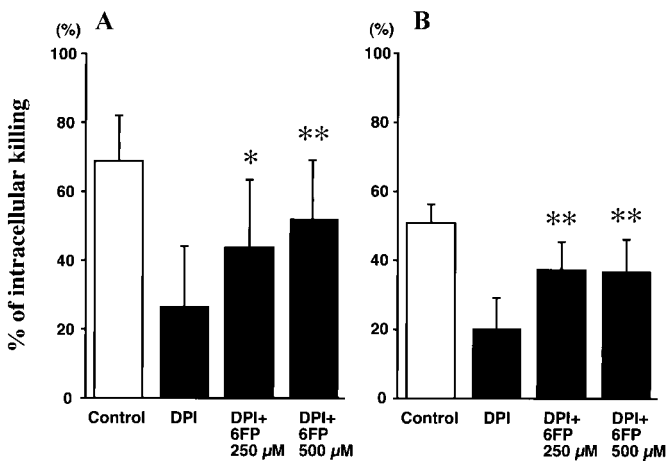

FIG. 4. Effects of 6-formylpterin on the bactericidal activity of neutrophils. The killing rates of neutrophils against *E. coli* (A) and *S. aureus* (B) are shown. The definition of the killing rate is shown in the text. In both A and B, Control, untreated neutrophils; DPI, the neutrophils preincubated with 10 μM diphenyleneiodonium; DPI + 6FP, the neutrophils preincubated with DPI and further incubated with 250 or 500 μM 6-formylpterin. The values are shown as means ± SD ($n = 10$ in A, $n = 5$ in B). *$P < 0.05$ and **$P < 0.01$, significantly different from the values obtained from the DPI neutrophils in each of A and B.

neutrophils significantly decreased to 20 ± 9% ($P < 0.001$ compared with untreated neutrophils). When DPI-treated neutrophils were incubated with 250 and 500 μM 6-formylpterin, the killing rates increased significantly to 38 ± 8% and to 37 ± 9% ($P < 0.05$ compared with DPI-treated neutrophils, in both) (Fig. 4B).

These results indicated that DPI impairs the bactericidal activity of neutrophils and that 6-formylpterin restores the impaired bactericidal activity of DPI-treated neutrophils.

DISCUSSION

In the present study, we demonstrated that 6-formylpterin intracellularly generated H_2O_2 in neutrophils, that the H_2O_2 generation was independent from NADPH-oxidase and that the generated H_2O_2 restored the impaired bactericidal activity of the DPI-treated neutrophils, a mimic for CGD neutrophils.

The access of CGD neutrophils with the impaired bactericidal activity is difficult because of the rarity of CGD, around 1/200,000 births (23). As an alternative method, we inhibited the NADPH-oxidase with DPI and made a mimic for CGD neutrophils. DPI is an inhibitor of a wide range of flavoproteins, including NADPH-oxidase, nitric oxide synthase, and complex I within the mitochondrial electron transport chain (24). The disadvantage of using DPI is its lack of specificity. However, in the present study, the use of DPI reduced the killing rate of normal neutrophils against bacteria and the reduced killing rate was restored by 6-formylpterin, which indicated that DPI impaired the oxidative killing mechanism of neutrophils but did not induce irreversible damages to the neutrophils. Therefore, DPI offered a good mimic for CGD in this study.

E. coli and *S. aureus* are catalase-positive organisms and known to be predominant pathogens in patients with CGD (25). So, we chose these bacteria as targets of killing. Although the increase in intracellular H_2O_2 induced by 6-formylpterin improved the impaired bactericidal activity of DPI-treated neutrophils against these bacteria, the activity was not completely restored. It was shown that $O_2^{\cdot-}$ made a direct contribution to killing of *S. aureus* (26) and that the H_2O_2-MPO system was not markedly involved in killing of *E. coli* (27). The NADPH-oxidase substantially generates $O_2^{\cdot-}$, whereas 6-formylpterin generates H_2O_2 but not $O_2^{\cdot-}$, which may be the reason why 6-formylpterin could not completely restore the impaired bactericidal activity.

Curative therapy for CGD has not been established except for allogeneic bone marrow transplantation (BMT). However, allogeneic BMT is rarely used in CGD because of its associated risks and the difficulty in finding a suitable donor (28). The best hope for long-term cures are recently developed gene therapy and nonmyeloablative hematopoietic stem cell transplantation (29, 30). Correction of only several percent of phagocytes enhances host defense in the murine model of CGD (31). Clinically, female carriers of the X-linked form of CGD with several percent of oxidase-positive neutrophils have few or no symptoms (32). These findings suggested that the incomplete restoration of the impaired bactericidal activity by 6-formylpterin may be sufficient for the enhancement of host defense and that the use of 6-formylpterin may be an alternative way to improve the bactericidal activity of the CGD patients. Not only in the CGD patients, but also in patients with human immunodeficiency virus (HIV) infection, it was reported that $O_2^{\cdot-}$ production of neutrophils was depressed and it may lead to the increased risk of serious bacterial infections in HIV-infected patients (33). Therefore, 6-formylpterin may contribute to enhance the bactericidal activity of neutrophils in HIV-infected patients.

In conclusion, we herein demonstrated that 6-formylpterin generated H_2O_2 independent from the NADPH-oxidase in human neutrophils and restored the impaired bactericidal activity of the DPI-treated neutrophils, a mimic for CGD. The ability of intracellular H_2O_2 production by 6-formylpterin may possibly serve to enhance innate immunity in patients with immunodeficient diseases.

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REFERENCES

1. Hampton, M. B., Kettle, A. J., and Winterbourn, C. C. (1998) Inside the neutrophil phagosome: Oxidants, myeloperoxidase, and bacterial killing. *Blood* **92,** 3007–3017.
2. Clark, R. A. (1999) Activation of the neutrophil respiratory burst oxidase. *J. Infect. Dis.* **179 Suppl. 2,** S309–S317.
3. Babior, B. M. (1999) NADPH oxidase: An update. *Blood* **93,** 1464–1476.
4. Lekstrom-Himes, J. A., and Gallin, J. I. (2000) Immunodeficiency diseases caused by defects in phagocytes. *N. Engl. J. Med.* **343,** 1703–1714.
5. Goldblatt, D., and Thrasher, A. J. (2000) Chronic granulomatous disease. *Clin. Exp. Immunol.* **122,** 1–9.
6. Mandell, G. L., and Hook, E. W. (1969) Leukocyte bactericidal activity in chronic granulomatous disease: Correlation of bacterial hydrogen peroxide production and susceptibility to intracellular killing. *J. Bacteriol.* **100,** 531–532.
7. Rex, J. H., Bennett, J. E., Gallin, J. I., Malech, H. L., and Melnick, D. A. (1990) Normal and deficient neutrophils can cooperate to damage *Aspergillus fumigatus hyphae. J. Infect. Dis.* **162,** 523–528.
8. Hampton, M. B., and Orrenius, S. (1997) Dual regulation of caspase activity by hydrogen peroxide: Implications for apoptosis. *FEBS Lett.* **414,** 552–556.
9. Kalckar, H. M., Kjeldgaard, N. O., and Klenow, H. (1948) Inhibition of xanthine oxidase and related enzymes by 6-pteridyl aldehyde. *J. Biol. Chem.* **174,** 771–772.

10. Lowry, O. H., Bessey, O. A., and Crawford, E. J. (1949) Pterine oxidase. *J. Biol. Chem.* **180,** 399–410.
11. Halpern, R., Halpern, B. C., Stea, B., Dunlap, A., Conklin, K., Clark, B., Ashe, H., Sperling, L., Halpern, J. A., Hardy, D., and Smith, R. A. (1977) Pterin-6-aldehyde, a cancer cell catabolite: Identification and application in diagnosis and treatment of human cancer. *Proc. Natl. Acad. Sci. USA* **74,** 587–591.
12. Arai, T., Endo, N., Yamashita, K., Sasada, M., Mori, H., Ishii, H., Hirota, K., Makino, K., and Fukuda, K. (2001) 6-Formylpterin, a xanthine oxidase inhibitor, intracellularly generates reactive oxygen species involved in apoptosis and cell proliferation. *Free Radic. Biol. Med.* **30,** 248–259.
13. Ellis, J. A., Mayer, S. J., and Jones, O. T. (1988) The effect of the NADPH oxidase inhibitor diphenyleneiodonium on aerobic and anaerobic microbicidal activities of human neutrophils. *Biochem. J.* **251,** 887–891.
14. Hampton, M. B., and Winterbourn, C. C. (1995) Modification of neutrophil oxidant production with diphenyleneiodonium and its effect on bacterial killing. *Free Radic. Biol. Med.* **18,** 633–639.
15. Watanabe, K., Arai, T., Mori, H., Nakao, S., Suzuki, T., Tajima, K., Makino, K., and Mori, K. (1997) Pterin-6-aldehyde, an inhibitor of xanthine oxidase, has superoxide anion radical scavenging activity. *Biochem. Biophys. Res. Commun.* **233,** 447–450.
16. Takahashi, A., Yamamoto, K., Okuma, M., and Sasada, M. (1992) Transient calcium elevation in polymorphonuclear leukocytes triggered by thrombin-activated platelets. *Eur. J. Haematol.* **48,** 196–201.
17. Royall, J. A., and Ischirooulos, H. (1993) Evaluation of 2′,7′-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular H2O2 in cultured endothelial cells. *Arch. Biochem. Biophys.* **302,** 348–355.
18. Zhou, M., and Panchuk-Voloshina, N. (1997) A one-step fluorometric method for the continuous measurement of monoamine oxidase activity. *Anal. Biochem.* **253,** 169–174.
19. Johnston, R. B., Jr., Keele, B. B., Jr., Misra, H. P., Leymeyer, J. E., Webb, L. S., Baehner, R. L., and RaJagopalan, K. V. (1975) The role of superoxide anion generation in phagocytic bactericidal activity. Studies with normal and chronic granulomatous disease leukocytes. *J. Clin. Invest.* **55,** 1357–1372.
20. Buettner, G. R. (1987) Spin trapping: ESR parameters of spin adducts. *Free Radic. Biol. Med.* **3,** 259–303.
21. Dunford, H. B. (1987) Free radicals in iron-containing systems. *Free Rad. Biol. Med.* **3,** 405–421.
22. Rota, C., Chignell, C. F., and Mason, R. P. (1999) Evidence for free radical formation during the oxidation of 2′-7′-dichlorofluorescin to the fluorescent dye 2′-7′-dichlorofluorescein by horseradish peroxidase: Possible implications for oxidative stress measurements. *Free Radic. Biol. Med.* **27,** 873–881.
23. Johnston, R. B., Jr. (2001) Clinical aspects of chronic granulomatous disease. *Curr. Opin. Hematol.* **8,** 17–22.
24. Li, Y., and Trush, M. A. (1998) Diphenyleneiodonium, an NAD(P)H oxidase inhibitor, also potently inhibits mitochondrial reactive oxygen species production. *Biochem. Biophys. Res. Commun.* **253,** 295–299.
25. Pitt, J., and Bernheimer, H. P. (1974) Role of peroxide in phagocytic killing of pneumococci. *Infect. Immun.* **9,** 48–52.
26. Hampton, M. B., Kettle, A. J., and Winterbourn, C. C. (1996) Involvement of superoxide and myeloperoxidase in oxygen-dependent killing of *Staphylococcus aureus* by neutrophils. *Infect. Immun.* **64,** 3512–3517.
27. Rosen, H., and Michel, B. R. (1997) Redundant contribution of myeloperoxidase-dependent systems to neutrophil-mediated killing of *Escherichia coli*. *Infect. Immun.* **65,** 4173–4178.
28. Kume, A., and Dinauer, M. C. (2000) Gene therapy for chronic granulomatous disease. *J. Lab. Clin. Med.* **135,** 122–128.
29. Malech, H. L. (1999) Progress in gene therapy for chronic granulomatous disease. *J. Infect. Dis.* **179 Suppl. 2,** S318–S325.
30. Horwitz, M. E., Barrett, A. J., Brown, M. R., Carter, C. S., Childs, R., Gallin, J. I., Holland, S. M., Linton, G. F., Miller, J. A., Leitman, S. F., Read, E. J., and Malech, H. L. (2001) Treatment of chronic granulomatous disease with nonmyeloablative conditioning and a T-cell-depleted hematopoietic allograft. *N. Engl. J. Med.* **344,** 881–888.
31. Mardiney, M., 3rd, Jackson, S. H., Spratt, S. K., Li, F., Holland, S. M., and Malech, H. L. (1997) Enhanced host defense after gene transfer in the murine p47phox-deficient model of chronic granulomatous disease. *Blood* **89,** 2268–2275.
32. Mills, E. L., Rholl, K. S., and Quie, P. G. (1980) X-linked inheritance in females with chronic granulomatous disease. *J. Clin. Invest.* **66,** 332–340.
33. Pitrak, D. L., Bak, P. M., DeMarais, P., Novak, R. M., and Andersen, B. R. (1993) Depressed neutrophil superoxide production in human immunodeficiency virus infection. *J. Infect. Dis.* **167,** 1406–1410.