Oxygen Metabolism in Phagocytes of Leprotic Patients: Enhanced Endogenous Superoxide Dismutase Activity and Hydroxyl Radical Generation by Clofazimine

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We examined the generation of active oxygens (O2·−, H2O2, and OH·) and the superoxide dismutase (SOD) activity of polymorphonuclear leukocytes (PMNs) and monocytes from 14 leprotic patients manifesting a bacillary index above 2.2. Patients with disease of more than 4 years in duration showed significantly enhanced SOD activity and a decrease in O2·− and OH· production. The antileprotic agent, clofazimine, significantly increased the generation of OH· in a dose-dependent manner, with a subsequent decrease in H2O2, but had no effect on the SOD activity of the PMNs and monocytes. In medium containing FeSO4 or Fe2+-EDTA, the drug elevated OH· production markedly further. Phagocytic SOD in PMNs and monocytes of leprotic patients was both host and bacillus derived, because the presence of cyanide, to which human-derived cuprozinc SOD is susceptible, did not completely abrogate SOD activity. The difficulty in treating leprosy may be partly ascribable to decreased phagocytic OH· generation, which in leprosy patients is apparently due to the uptake of Hansen bacillus-derived SOD. Clofazimine may be effective in leprosy by chelating Fe2+, with the resultant potentiation of the catalyzing activity of Fe3+ in the Haber-Weiss reaction increasing OH· formation from H2O2.

The long-term survival rate of patients with leprosy has improved since the introduction into clinical use of the antileprotic agents dapsone (4,4′-diaminodiphenyl sulfone) and clofazimine [3-(p-chloroanilino)-10-(p-chlorophenyl)-2,10-dihydro-2-(isopropylimino)phenazine]. Both of these drugs are thought to act in leprosy and in nonleprotic diseases by affecting neutrophil function (13, 15, 19–21, 32–34, 36, 37). Patients with leprosy have been reported to show both diminished phagocytosis (3) and reduced phagocytic chemotaxis (30).

Although clofazimine has been reported to be effective in the treatment of diseases with defective phagocytosis (21, 33), experiments in our laboratory failed to demonstrate an effect of clofazimine on the phagocytic function of neutrophils in vitro. We reasoned, therefore, that clofazimine may enhance other functions of neutrophils in patients with leprosy. In the experiments described herein, we examined the neutrophil-derived generation of active oxygens (AO) and activity of superoxide dismutase (SOD) in patients with leprosy. We present data that are relevant to the pathogenesis of leprosy and the potential mechanism of action of clofazimine in the treatment of leprosy.

MATERIALS AND METHODS

Experimental subjects. Fourteen patients with histories of more than 4 years of leprosy (Hansen’s disease [HD]) were recruited from the National Leprosanatorium, Nagashima Aiseien, Okayama, Japan. All patients had a bacillary index (25) exceeding 2.2. Nine patients (seven males, aged 29 to 64, and two females, aged 48 and 54) had received 50 mg of dapsone per day for 2 to 3 years; five patients (four males, aged 47 to 61, and one female, aged 45) had received 100 mg of clofazimine twice a week for 2 to 3 years. All antileprotic drugs were withheld for 3 days before drawing blood. In five patients who were in the highly active state, i.e., with bacillary indices (25) above 4.0, neutrophilic and monocyte SOD activities were assayed.

Two age- and sex-matched control groups were studied. Group 1 consisted of 12 patients with bacterial infections (pneumonia, appendicitis, cholecystitis, pyelonephritis, and periarticular abscess), each of whom had been febrile (>39°C) for more than 5 days and showed leukocytosis and neutrophilia (leukocytes, >10,000/mm3; neutrophils, >70%). Control group 2 consisted of 10 healthy volunteers.

Preparation of PMNs or monocytes. Using a previously described technique (24), polymorphonuclear leukocytes (PMNs) were isolated from peripheral blood and suspended in Krebs-Ringer phosphate buffer (KRP) (7) containing glucose (5 mM) and gelatin (1 mg/ml) for assays of AO and lysozymatic enzyme generation and SOD activity. KRP containing only 5 mM glucose was used for the OH· generation assay. Mononuclear cell fractions (including lymphocytes and monocytes) were obtained at the same time as PMNs. Adherent cells (monocytes) were separated from the mononuclear cell fractions by incubation on petri dishes at 37°C for 2 h in 5% CO2. Giemsa staining and reaction with OM one, a monoclonal antibody (Ortho Pharmaceutical Corp., Raritan, N.J.) that binds specifically to monocytes and cells of the myeloid series, confirmed that 95% of the cells in the monocyte preparations were monocytes. These cells were used for SOD activity assays and for determination of the effect of KCN on this activity.

AO generation assays. PMNs were assayed for AO generation as previously described (23, 24). Briefly, O2·− formation was determined by assessing ferricytochrome c reduction by O2·− produced from 4 × 10⁶ PMNs stimulated with 1 mg of opsonized zymosan (Sigma Chemical Co.) per ml measured at 550 nm absorbance. H2O2 generation was determined by using 2.5 × 10⁶ PMNs stimulated with 1 mg of opsonized zymosan per ml, 0.1 ml of 50 mM scoleptic in KRP, and 0.1 ml of horseradish peroxidase at a concentration of 1 mg/ml;
the rate of decrease in fluorescent intensity of the scopoletin within 30 min was quantitated in a fluorescence spectrophotometer. OH· was quantitated by the amount of ethylene gas formed from α-keto-methyl butylic acid and $2 \times 10^6$ PMNs stimulated with 1 mg of opsonized zymosan per ml; the total amount of OH· formed at 10, 20, and 30 min was determined on a gas chromatograph.

The specificity of each assay was confirmed by the depletion of each AO by its specific corresponding scavenger (400 U of SOD per ml for $O_2^-$, 600 U of catalase per ml for $H_2O_2$, and 10 mM benzoate for OH·). Since ethylene formation from α-keto-methyl butylic acid can potentially be mediated by radicals other than OH·, the specificity of the OH· assay was confirmed by another assay method (38) simultaneously with the ethylene formation method. The formaldehyde formation method from Me₃SO and tert-butyl alcohol, as well as the N-dimethylation of aminopyrine (6), was determined by the method of Nash (22). OH· generation by both methods showed similar behavior. SOD and catalase heated at 130°C for 30 min had no effect on the generation of $O_2^-$ and $H_2O_2$, respectively. In each AO assay system, PMNs were stimulated by opsonized zymosan were simultaneously tested.

**SOD activity assay.** First, PMNs or monocytes were sonified and suspended in KRP for use in the assay of SOD activity. Then, $O_2^-$ was generated by the xanthine-xanthine oxidase system; 0.05 ml of 0.66 mM ferricytochrome c and 0.1 ml of 2 mM hypoxanthine in 50 ml of physiological saline plus 0.05 ml of 50 mM EDTA in 2.3 ml of physiological saline were mixed. Thereafter, 0.1 ml of this mixture was diluted in 1 ml of KRP (pH 7.2 to 7.4), and then 1 ml of SOD-containing fluids from $2 \times 10^6$ PMNs or monocytes was added. Finally, 0.1 ml of dialyzed xanthine oxidase at a concentration of 0.1 U/ml was added to generate $O_2^-$. Under these conditions, the amount of SOD required to inhibit the rate of reduction of cytochrome c by 50% (i.e., to a rate of 0.0125 absorbance unit per min) was defined as 1 U of activity (18).

**Characterization of phagocytic SOD.** KCN, 0.1, 0.5, or 1 mM, in 100 mM potassium phosphate, pH 7.8, was introduced into the assay system for SOD activity in PMNs or monocyte fluids from leproptic patients or healthy controls. The enzymatic activity was determined (2) after the chemicals were removed by dialysis; alternatively, small portions were used. SOD activities in PMN- or monocyte-containing fluids from HD patients and healthy controls were compared.

**Effect of clofazimine on AO generation and SOD activity.** To determine the dose-response effect, clofazimine (0.01, 0.1, or 1 mM) was dissolved in 95% ethanol and added in a final volume of 1% to assay AO generation and SOD activity in PMNs, monocytes, or both from healthy controls. In these experiments, the same volume of 95% ethanol was added, and the AO levels or SOD activity in the presence and absence of clofazimine were compared.

**Effect of clofazimine on AO generation in the presence of iron-containing drugs.** First, to evaluate the dose-response effect, clofazimine, FeSO₄, or FeCl₂·EDTA (each at a concentration of 0.01, 0.1, or 1 mM) was added to the assay medium to assess AO generation in PMNs from the healthy controls. Thereafter, checkerboard experiments were performed; various concentrations (0.01, 0.1, or 1 mM) of each agent were added to the AO-generating system in which each different agent at a fixed concentration of 1 mM was present. By this procedure, the dose-dependent effect of each agent on AO generation affected by each different agent at a fixed concentration was determined. ($O_2^-$ generation alone could not be evaluated in this experiment because iron-containing chemicals reduce directly ferricytochrome c.)

**AO generation assay in the xanthine-xanthine oxidase system.** To confirm the effect of clofazimine on AO generation in the presence of iron-containing drugs, the effect was also examined by producing all AO in the xanthine-xanthine oxidase system. Instead of adding PMNs and opsonized zymosan, 0.1 ml of 2 mM hypoxanthine in 50 ml of physiological saline plus 0.05 ml of 50 mM EDTA were diluted in 2 ml of KRP (pH 7.2 to 7.4). Thereafter, 0.1 ml of dialyzed xanthine oxidase at a concentration of 0.1 U/ml was added to generate $O_2^-$. Taking into consideration the inhibitory effect of hypoxanthine on AO production in this assay system, we also substituted acetaldehyde (Nakarai Chemicals, Kyoto, Japan) for the hypoxanthine substrate.

**Viability and phagocytic function of PMNs.** The viability of PMNs after their incubation with clofazimine was determined by means of the trypan blue dye exclusion test and was uniformly 100%; phagocytic function was evaluated by the zymosan-stimulated uptake of $[^{14}C]$inulin (7). No PMN preparation showed a $[^{14}C]$inulin uptake of less than 600 dpm of protein per mg, indicating that the drug per se did not impair PMN function (7, 24).

**Statistical analysis.** Triplicate assays were performed simultaneously for each experiment and the results were expressed as the mean ± standard error. The statistical significance was ascertained by the Student t test.

**RESULTS**

**AO generation.** Compared with healthy controls, PMNs from HD patients generated markedly less OH· (P < 0.01) and significantly less $O_2^-$ (0.025 < P < 0.05). The generation of $H_2O_2$ was lower in HD patients, but the difference from controls was not statistically significant (P > 0.05) (Table 1). Although some of the patients with bacterial infections manifested increased levels of $O_2^-$, $H_2O_2$, and OH·, there was no significant difference between them and the healthy

**TABLE 1. Generation of AO by zymosan-stimulated PMNs from HD patients and control groups**

<table>
<thead>
<tr>
<th>AO</th>
<th>Mean ± SEM pmol x 10⁷/min generated by:</th>
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<tbody>
<tr>
<td></td>
<td>HD patients (n = 14)</td>
</tr>
<tr>
<td></td>
<td>With zymosan</td>
</tr>
<tr>
<td>$O_2^-$ ($4 \times 10^6$ PMNs)</td>
<td>3.9 ± 0.22$^a$</td>
</tr>
<tr>
<td>$H_2O_2$ ($2.5 \times 10^6$ PMNs)</td>
<td>3.68 ± 0.321</td>
</tr>
<tr>
<td>OH· ($2 \times 10^6$ PMNs)</td>
<td>5.1 ± 0.182$^b$</td>
</tr>
<tr>
<td></td>
<td>Healthy controls (n = 6)</td>
</tr>
<tr>
<td></td>
<td>With zymosan</td>
</tr>
<tr>
<td>$O_2^-$ ($4 \times 10^6$ PMNs)</td>
<td>5.0 ± 0.15</td>
</tr>
<tr>
<td>$H_2O_2$ ($2.5 \times 10^6$ PMNs)</td>
<td>4.11 ± 0.165</td>
</tr>
<tr>
<td>OH· ($2 \times 10^6$ PMNs)</td>
<td>9.8 ± 0.232</td>
</tr>
<tr>
<td></td>
<td>Infected controls (n = 12)</td>
</tr>
<tr>
<td></td>
<td>With zymosan</td>
</tr>
<tr>
<td>$O_2^-$ ($4 \times 10^6$ PMNs)</td>
<td>6.7 ± 1.56</td>
</tr>
<tr>
<td>$H_2O_2$ ($2.5 \times 10^6$ PMNs)</td>
<td>6.51 ± 1.36</td>
</tr>
<tr>
<td>OH· ($2 \times 10^6$ PMNs)</td>
<td>13.9 ± 2.58</td>
</tr>
</tbody>
</table>

$^a$ 0.025 < P < 0.05.
$^b$ P < 0.001 versus healthy controls.
controls (P > 0.05) on account of a large standard error of the mean in bacteria-infected patients.

SOD activity. Compared with the healthy controls, SOD activity in HD PMNs was slightly increased (0.025 < P < 0.05), and in monocytes it was highly increased (P < 0.01) (Table 2). The activities in PMNs and monocytes from the patients with bacterial infections were similar to those of the healthy controls (data not shown).

Characterization of phagocytic SOD. The addition of KCN decreased SOD activity in a dose-dependent fashion in PMNs and monocytes from HD patients and healthy controls. However, at each concentration of KCN, SOD activity in both cell types from HD patients was greater than that of controls (Fig. 1), suggesting the presence of bacteria-derived, KCN-resistant SOD in the phagocytes from HD patients. However, there still exists the possibility that in addition to bacteria-derived SOD, mitochondria-derived SOD may also be present, although mitochondria-derived SOD seems to be negligible because of a very small amount of SOD in mitochondria.

Effect of clofazimine on AO generation and SOD activity.
Clofazimine did not affect the SOD activity of either PMNs or monocytes from healthy individuals (data not shown). In the absence of Fe²⁺-EDTA or FeSO₄, clofazimine caused, in a dose-dependent manner, a significant decrease in the generation of H₂O₂ and a significant increase in the generation of O₂⁻ and OH⁻ by PMNs of normal individuals (all AOs with 1 mM; P < 0.01) (Fig. 2).

Effect of clofazimine on AO generation in the presence of FeSO₄ and Fe²⁺-EDTA. Among FeSO₄, Fe²⁺-EDTA, and clofazimine, clofazimine was the most effective in potentiating OH⁻ generation and in attenuating the production of H₂O₂ (Figs. 2 to 4). In the presence of FeSO₄ or Fe²⁺-EDTA at a fixed concentration of 1 mM, clofazimine also markedly increased OH⁻ generation and decreased H₂O₂ production. This marked increase in OH⁻ and decrease in H₂O₂ was induced in a dose-dependent manner by clofazimine and was far greater than both the changes induced by the single addition of FeSO₄ or Fe²⁺-EDTA and those observed with the simultaneous addition of FeSO₄ and Fe²⁺-EDTA. All of these changes were observed by the catalytic amounts of each drug. Similar results were also obtained in the experiment concerning the effect of clofazimine on AO generation by the xanthine-xanthine oxidase system in the presence of iron-containing drugs (data not shown).

These observations seem to suggest that OH⁻ formation from H₂O₂ catalyzed by Fe²⁺ with a concurrent decrease in H₂O₂ (in the Fenton-type reaction) was accelerated by clofazimine. There is also the possibility that this potentiated catalyzing activity of Fe²⁺ was induced by the chelation of iron by clofazimine. It is likely that iron clofazimine is an effective catalysis for the Haber-Weiss reaction to produce OH⁻ radicals from H₂O₂. Our hypothesis about the chelation of iron by clofazimine is supported by the fact that similar findings obtained in cellular systems were also seen in simple experimental systems such as the xanthine oxidase system. The fact that clofazimine alone showed the same qualitative effects suggests that some amounts of Fe²⁺ are present in PMNs.

When we used acetaldehyde rather than hypoxanthine as

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**TABLE 2. SOD activity in PMNs and monocytes from HD patients and healthy controls**

<table>
<thead>
<tr>
<th>Sample</th>
<th>SOD activity (U/2 × 10⁶ cells)</th>
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<tbody>
<tr>
<td>PMNs from patients (n = 5)</td>
<td>0.60 ± 0.054α</td>
</tr>
<tr>
<td>PMNs from controls (n = 6)</td>
<td>0.48 ± 0.043</td>
</tr>
<tr>
<td>Monocytes from patients (n = 5)</td>
<td>1.08 ± 0.025b</td>
</tr>
<tr>
<td>Monocytes from controls (n = 6)</td>
<td>0.64 ± 0.049</td>
</tr>
</tbody>
</table>

α 0.025 < P < 0.05.
β P < 0.01 versus the healthy controls.

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**FIG. 1.** Effect of cyanide on SOD activity in PMNs or monocytes from HD patients and healthy controls. † Control contains the same volume of 95% ethanol which is used to dissolve the drug in PMN medium.

**FIG. 2.** Effect of clofazimine on AO generation by PMNs from healthy individuals. † See the legend to Fig. 1. (a) Ferricytochrome c reduction (pmol/10⁶/min per 4 × 10⁶ PMNs); (b) H₂O₂ generation (pmol × 10⁶/min per 2.5 × 10⁶ PMNs); and (c) ethylene formation (pmol × 10⁶/2 × 10⁶ PMNs).
the substrate in the xanthine oxidase system, we obtained comparable results (not shown), thereby ruling out the possibility of an inhibitory effect of hypoxanthine on AO generation.

**DISCUSSION**

We have observed that PMNs from HD patients show lower O$_2^-$ and OH$^-$ production (Table 1) and higher SOD activity than do those of healthy individuals (Table 2). These observed abnormalities are probably causally related, because enhancement of SOD activity diminishes the steady-state concentration of O$_2^-$, leading to decreased OH$^-$ production due to the inhibition of the reduction of Fe$^{3+}$ to Fe$^{2+}$ (1, 28).

The fact that OH$^-$ production was lower than O$_2^-$ production (Table 1) may reflect the effect of dapsone therapy which reduces OH$^-$ generation with a resultant slight increase in O$_2^-$ (23a). The concentration of H$_2$O$_2$ was not increased significantly, despite the potentiation in SOD activity, possibly because H$_2$O$_2$ has various disposal pathways, including a rapid catalase-mediated scavenging.

The enhancement of SOD activity in HD patients observed in this study may be ascribable to the high level of SOD in Hansen bacillus (12, 14); the increased SOD activity in phagocytes from leproptic patients is probably due to phagocytosis of *Mycobacterium leprae*, which contains a large amount of SOD (12, 14). It may be argued that bacteria-derived SOD in patient phagocytes is inactive. However, based on the following considerations, we contend that this objection is invalid. (i) Manganese SOD, an enzyme of bacterial origin, is resistant to H$_2$O$_2$ (2) which attacks foreign particles or invading agents in phagosomes. (ii) Our total activity assay revealed that SOD activity remained even after the addition of KCN which inhibits copper-zinc SOD, a human-origin enzyme (2, 8). These facts suggest that in HD patient phagocytes, SOD of both bacterial and human origin is present. Furthermore, peripheral blood monocytes have a much longer lifespan than do PMNs, suggesting that monocytes phagocytize and contain a greater amount of SOD than do PMNs (4). Therefore, the greater SOD activity in monocytes than in PMNs from HD patients (Table 2) supports our hypothesis that in such patients, the enhanced phagocytic SOD activity is ascribable to the phagocytosis of the Hansen bacillus. In support of this hypothesis, Stach et al. (32) reported marked enhancement of SOD activity in mice infected with Hansen bacillus. Actually, it is well known that staphylococcal infection has a lethal effect in patients with chronic granulomatous disease, because PMNs from such patients are deficient in generating AO to kill staphylococci, which is exacerbated by the uptake of staphylococci containing a large amount of catalase (16). A similar pathogenetic process can be proposed for patients with leprosy.

The decreased generation of OH$^-$ and O$_2^-$ in leproptic patients observed in this study also seems to be derived from...
enhanced SOD activity. We initially expected an inhibitory effect of clofazimine on SOD activity, but the drug was not found to affect SOD activity. In view of the data obtained in the present study, in the medium supplemented with a ferrous ion, clofazimine more markedly potentiated OH· production and attenuated the generation of H2O2 than it did in the absence of FeSO4 and Fe2+-EDTA. Clofazimine seems to potentiate the catalyzing activity of the ferrous ion, resulting in an increase in OH· production. Recently, Rosen and Klebanoff documented the catalysis of the Haber-Weiss reaction in Fe2+-EDTA (28). In our laboratory, we observed that the addition of clofazimine to FeSO4-containing medium turns the color of the medium pink, suggesting the formation of an iron-chelator complex. Thereafter, we compared the absorption spectrum between clofazimine and Fe2+, the result of which confirmed the validity of the chelation of iron by clofazimine (unpublished data). This further supports the concept that clofazimine chelates the ferrous ion, leading to enhancement of the catalytic activity of Fe2+ to produce OH· from H2O2 by the Haber-Weiss reaction. Thus, the drug probably effectively restores disturbed phagocytic functions in HD patients by reversing the decrease in phagocyte OH· levels induced by the enhanced bacillus-derived SOD activity.

However, there is still the possibility that the decreased generation of OH· and O2·− seen in HD patients is due to a diminished oxygen burst of PMNs from HD patients, because all of the AO assessed showed the tendency toward lower levels (Table 1). In addition, the small increase in SOD measured in PMNs from patients (Table 2) suggests that the findings observed in HD patients in the present study are partly ascribable to their intrinsically decreased AO generation by HD patients.

Previous studies of lepromatous patients have reported the reduction of phagocytosis (3), decreased chemotaxis (30), lowered PMN mobilization (5), serum factors that inactivate leucotransfactors (35), and normal lysosomal enzyme levels (10). In contrast to our findings, Sher et al. (30) and Rojas-Espinosa (26) have reported normal levels of O2− and normal tetrazolium salts reduction in HD patients. The discrepancy between these findings and ours may be explained by our discontinuing treatment before testing each patient.

In summary, we postulate that phagocytosis of the Hansen bacillus leads to defective phagocyte function, at least partly because of the activity of bacillus-derived SOD, resulting in the incurability of the disease. Furthermore, decreased generation of OH· (Table 1), an active and potent AO in both bacterial and cytotoxic activity (11, 17, 27, 29), seems to exacerbate the attenuation of the self-defense mechanism in lepromatous patients. The pathogenesis of leprosy appears to be at least partially related to enhanced phagocytic SOD activity and to a defect in phagocytic function. Clofazimine may be effective in the treatment of lepromatous patients by increasing OH· generation through the catalytic action of the iron clofazimine-chelating complex.

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LITERATURE CITED


