JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1984, p. 837–842
0095-1137/84/110837-06$02.00/0

Vol. 20, No. 5

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

YUKIE NIWA,[1]* TSUYOSHI SAKANE,[1] YOSHIKI MIYACHI,[2] AND MOTOAKI OZAKI[2]

Third Department of Internal Medicine, Shimane Medical University, Shimane 693,[1] and Department of Dermatology, Kyoto University, Kyoto 606,[2] Japan

Received 19 March 1984/Accepted 14 July 1984

We examined the generation of active oxygens (O_2^-, H_2O_2, 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 O_2^- and OH · production. The antileprotic agent, clofazimine, significantly increased the generation of OH · in a dose-dependent manner, with a subsequent decrease in H_2O_2, but had no effect on the SOD activity of the PMNs and monocytes. In medium containing $FeSO_4$ or Fe^{2+}-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 Fe^{2+}, with the resultant potentiation of the catalyzing activity of Fe^{2+} in the Haber-Weiss reaction increasing OH · formation from H_2O_2.

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 monocytic 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 peritonsillar abscess), each of whom had been febrile (>39°C) for more than 5 days and showed leukocytosis and neutrophilia (leukocytes, >10,000/mm^3; 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 lysosomal 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% CO_2. 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, O_2^- formation was determined by assessing ferricytochrome *c* reduction by O_2^- produced from 4×10^6 PMNs stimulated with 1 mg of opsonized zymosan (Sigma Chemical Co.) per ml measured at 550 nm absorbance. H_2O_2 generation was determined by using 2.5×10^6 PMNs stimulated with 1 mg of opsonized zymosan per ml, 0.1 ml of 50 mM scopoletin in KRP, and 0.1 ml of horseradish peroxidase at a concentration of 1 mg/ml;

* Corresponding author.

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-methiol butylic acid and 2×10^6 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-methiol 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 Me_2SO and *t*-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 not 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 2×10^6 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 leprotic 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, $FeSO_4$, or Fe^{2+}-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 (9).

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

AO	Mean ± SEM pmol × 10^2/min generated by:					
	HD patients (n = 14)		Healthy controls (n = 6)		Infected controls (n = 12)	
	With zymosan	Without zymosan	With zymosan	Without zymosan	With zymosan	Without zymosan
O_2^- (4×10^6 PMNs)	3.9 ± 0.22[a]	0.16 ± 0.04[a]	5.0 ± 0.15	0.21 ± 0.03	6.7 ± 1.56	0.25 ± 0.059
H_2O_2 (2.5×10^6 PMNs)	3.68 ± 0.321	0.98 ± 0.06	4.11 ± 0.165	1.24 ± 0.09	6.51 ± 1.36	1.70 ± 0.49
OH · (2×10^6 PMNs)	5.1 ± 0.182[b]	0.12 ± 0.007[b]	9.8 ± 0.232	0.183 ± 0.015	13.9 ± 2.58	0.23 ± 0.046

[a] $0.025 < P < 0.05$.
[b] $P < 0.001$ versus healthy controls.

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

Sample	SOD activity (U/2 × 10^6 cells)
PMNs from patients (n = 5)	0.60 ± 0.054[a]
PMNs from controls (n = 6)	0.48 ± 0.043
Monocytes from patients (n = 5)	1.08 ± 0.025[b]
Monocytes from controls (n = 6)	0.64 ± 0.049

[a] $0.025 < P < 0.05$.
[b] $P < 0.01$ versus the 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 Fe^{2+}-EDTA or $FeSO_4$, clofazimine caused, in a dose-dependent manner, a significant decrease in the generation of H_2O_2 and a significant increase in the generation of O_2^- and OH · by PMNs of normal individuals (all AOs with 1 mM; $P < 0.01$) (Fig. 2).

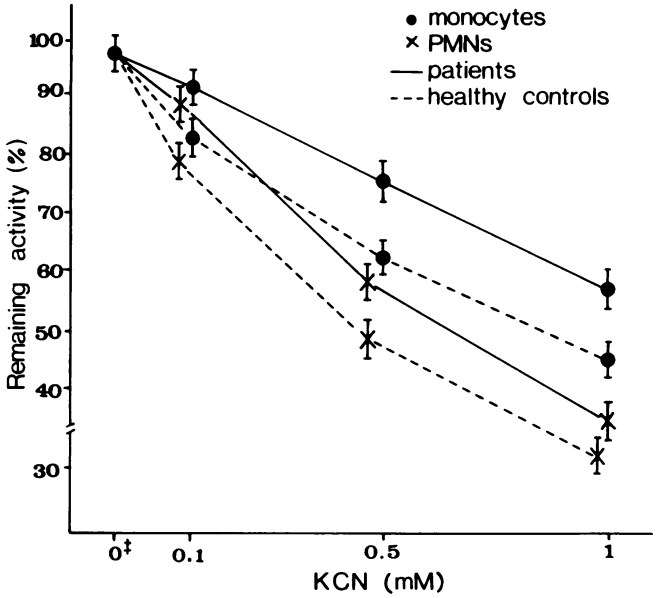

FIG. 1. Effect of cyanide on SOD activity in PMNs or monocytes from HD patients and healthy controls. ‡, Control contains the same volume of 95% ethanol which is used to dissolve the drug in PMN medium.

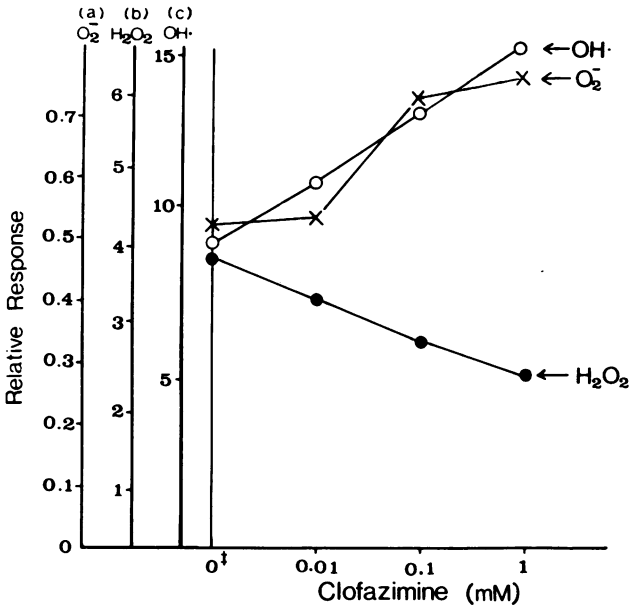

FIG. 2. Effect of clofazimine on AO generation by PMNs from healthy individuals. ‡, See the legend to Fig. 1. (a) Ferricytochrome c reduction (pmol × 10^2/min per 4 × 10^6 PMNs); (b) H_2O_2 generation (pmol × 10^2/min per 2.5 × 10^6 PMNs); and (c) ethylene formation (pmol × 10^2/2 × 10^6 PMNs).

Effect of clofazimine on AO generation in the presence of $FeSO_4$ and Fe^{2+}-EDTA. Among $FeSO_4$, Fe^{2+}-EDTA, and clofazimine, clofazimine was the most effective in potentiating OH · generation and in attenuating the production of H_2O_2 (Figs. 2 to 4). In the presence of $FeSO_4$ or Fe^{2+}-EDTA at a fixed concentration of 1 mM, clofazimine also markedly increased OH · generation and decreased H_2O_2 production. This marked increase in OH · and decrease in H_2O_2 was induced in a dose-dependent manner by clofazimine and was far greater than both the changes induced by the single addition of $FeSO_4$ or Fe^{2+}-EDTA and those observed with the simultaneous addition of $FeSO_4$ and Fe^{2+}-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 H_2O_2 catalyzed by Fe^{2+} with a concurrent decrease in H_2O_2 (in the Fenton-type reaction) was accelerated by clofazimine. There is also the possibility that this potentiated catalyzing activity of Fe^{2+} 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 H_2O_2. 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 Fe^{2+} are present in PMNs.

When we used acetaldehyde rather than hypoxanthine as

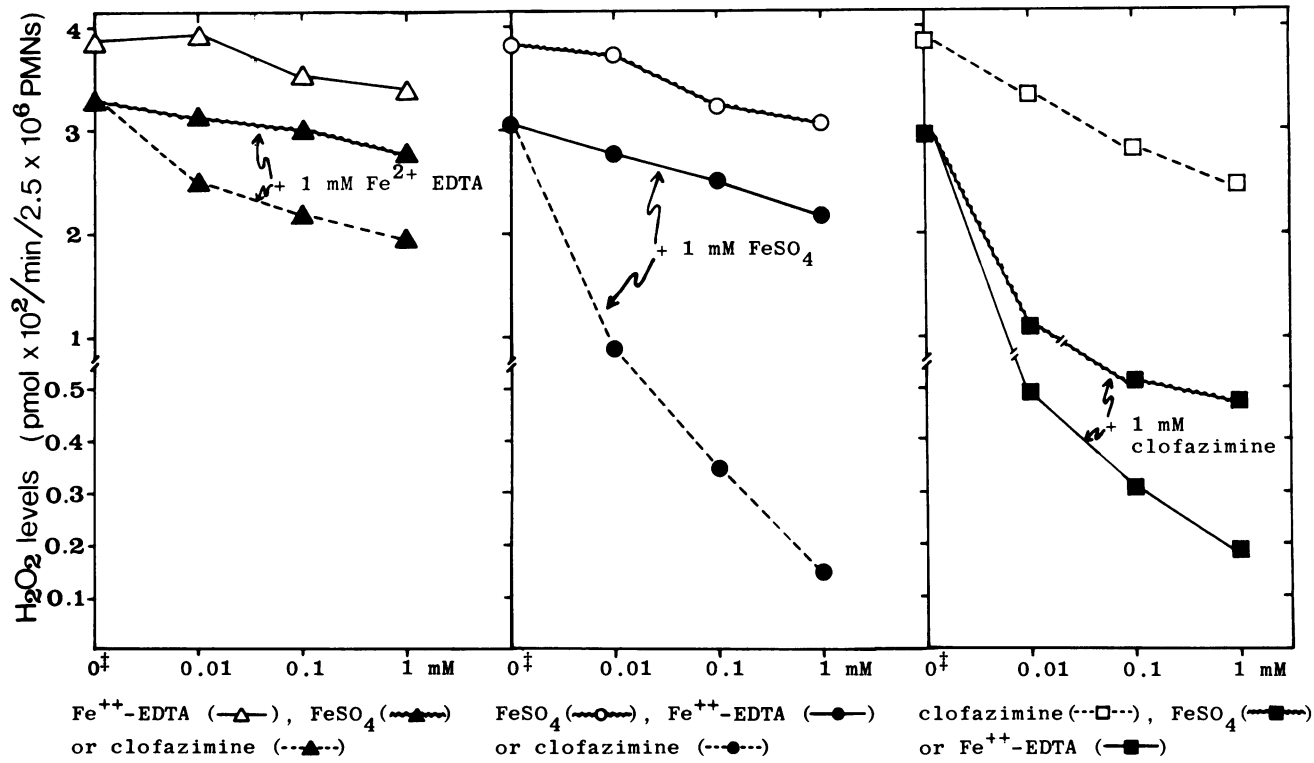

FIG. 3. Comparison of effects between clofazimine and iron-containing agents on H_2O_2 generation in the presence of iron-containing drugs and clofazimine. First, to evaluate the dose-response effect of each agent alone, clofazimine, $FeSO_4$, or Fe^{2+}-EDTA (each at a concentration of 0.01, 0.1, or 1 mM) was added to the assay medium to assess AO generation in PMNs from healthy individuals. Thereafter, checkerboard experiments were performed; in the presence of each agent at a fixed concentration of 1 mM in the AO-generating system, each other agent was further added in a dose-dependent fashion (each at a concentration of 0.01, 0.1, or 1 mM), respectively. The dose-dependent effect of each agent on AO generation affected by a different agent at a fixed concentration was determined and compared. For example, the left-hand figure represents the dose-dependent effect by Fe^{2+}-EDTA itself on AO generation in PMN medium and that of $FeSO_4$ or clofazimine on AO generation in PMN medium containing Fe^{2+}-EDTA fixed at 1 mM.

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_2O_2 was not increased significantly, despite the potentiation in SOD activity, possibly because H_2O_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 leprotic 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_2O_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 leprotic patients observed in this study also seems to be derived from

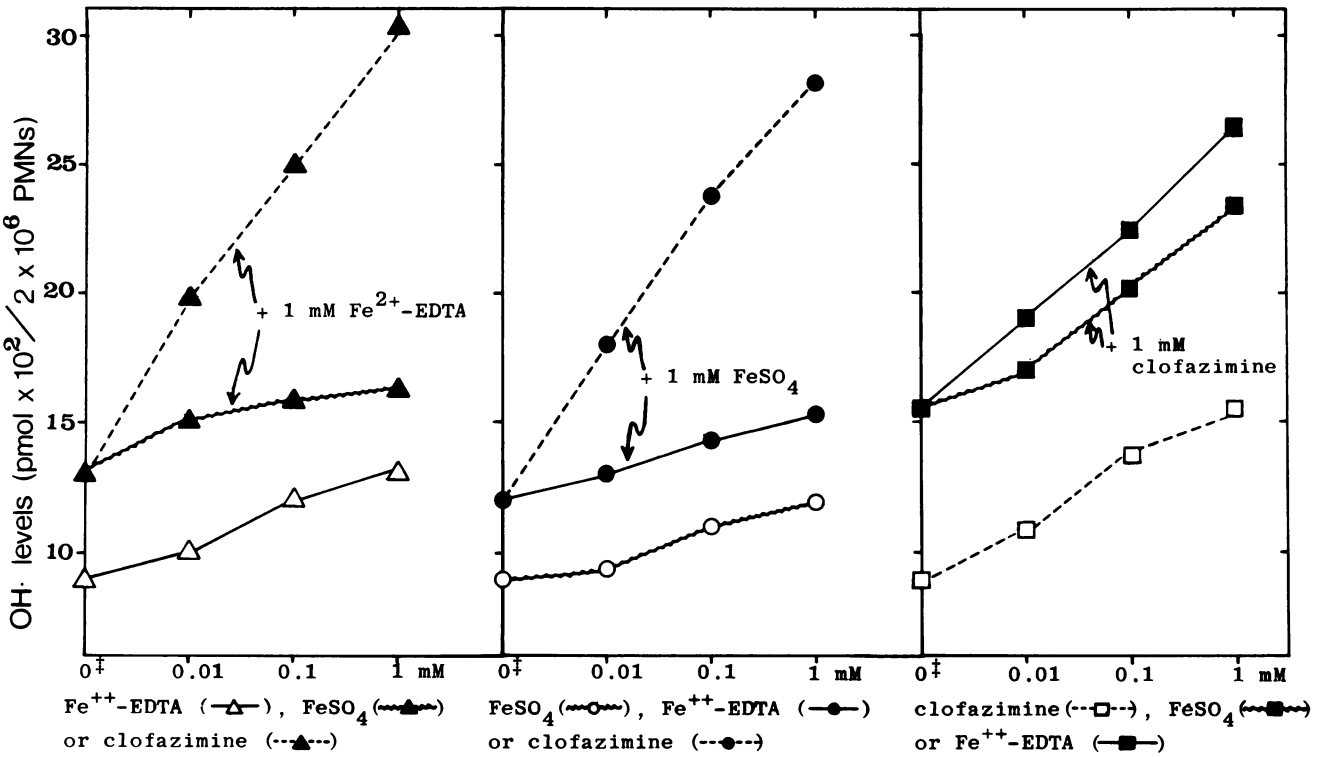

FIG. 4. Comparison of effects between clofazimine and iron-containing agents on OH · generation in the presence of iron-containing drugs and clofazimine. For explanation of symbols, see the legend to Fig. 3.

enhanced SOD activity. We initially expected an inhibitory effect of clofazimine on SOD activity, but the drug was found not 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 H_2O_2 than it did in the absence of $FeSO_4$ and Fe^{2+}-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 Fe^{2+}-EDTA (28). In our laboratory, we observed that the addition of clofazimine to $FeSO_4$-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 Fe^{2+}, 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 Fe^{2+} to produce OH · from H_2O_2 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 O_2^- 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 leprotic patients have reported the reduction of phagocytosis (3), decreased chemotaxis (30), lowered PMN mobilization (5), serum factors that inactivate leucoattractants (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 O_2^- 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 leprotic 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 leprotic patients by increasing OH · generation through the catalytic action of the iron clofazimine-chelating complex.

ACKNOWLEDGMENT

We are indebted to Kozi Asada, Research Institute for Food Science, Kyoto University, for expert assistance in the preparation of this manuscript.

LITERATURE CITED

1. **Ambruso, D. R., and R. B. Johnston, Jr.**, 1981. Lactoferrin enhances hydroxyl radical production by human neutrophils, neutrophil particulate fractions, and an enzymatic generating system. J. Clin. Invest. **67**:352–360.
2. **Asada, K., K. Yoshikawa, M. Takahashi, Y. Maeda, and K. Enmanji.** 1975. Superoxide dismutase from a blue-green alga,

Plectonema boryanum. J. Biol. Chem. **250**:2801–2807.
3. **Barbieri, T. A., and W. M. Correa.** 1967. Human macrophage culture: the leprosy prognostic test (LPT). Int. J. Lepr. **35**:377–381.
4. **Bechelli, L. M., G. Klingmüller, M. J. Quiroga, and H. Schmidt.** 1970. Infektionskrankheiten der haut II, p. 176–177. *In* J. Jadassohn (ed.), Handbuch der Hautund Geschlechtskrankheiten. Springer-Verlag, Berlin.
5. **Bullock, W. E., Jr., M. F. Ho, and M. J. Chen.** 1974. Qualitative and quantitative studies of the local cellular exudative response in leprosy. RES J. Reticuloendothel. Soc. **16**:259–268.
6. **Cochin, J., and J. Axelrod.** 1959. Biochemical and pharmacological changes in the rat following chronic administration of morphine, nalorphine and normorphine. J. Pharmacol. Exp. Ther. **125**:105–110.
7. **Cohen, P. P.** 1957. Suspending media for normal tissues, p. 149–150. *In* W. W. Umbreit, R. H. Burris, and J. F. Stauffer (ed.), Monometric techniques and tissue metabolism, 3rd ed. Burgess Publishing Co., Minneapolis.
8. **Crapo, J. D., J. M. McCord, and I. Fridovich.** 1978. Superoxide dismutase preparation and assay, import. Methods Enzymol. **52**:382–393.
9. **Fridovich, I.** 1970. Quantitative aspects of the production of superoxide anion radical by milk xanthine oxidase. J. Biol. Chem. **245**:4053–4057.
10. **Garcia-Gonzales, J. E., O. Rojas-Espinosa, and S. Estrada-Parra.** 1977. Phagocytosis in leprosy. I. The levels of "diaphorase", β-glucuronidase, acid phosphatase, alkaline phosphatase and lipase in circulating leukocytes. Lepr. Rev. **48**:17–26.
11. **Haber, F., and J. Weiss.** 1934. The catalytic decomposition of hydrogen peroxide by iron salts. Proc. R. Soc. Lond. A **147**:332–351.
12. **Ichihara, K., E. Kusunose, M. Kusunose, and T. Mori.** 1977. Superoxide dismutase from mycobacterium. J. Biochem. **81**:1427–1433.
13. **Kark, E. C., B. R. Davis, and J. R. Pomeranz.** 1981. Pyoderma gangrenosum treated with clofazimine. Am. Acad. Dermatol. **4**:152–159.
14. **Kusunose, M., Y. Noda, K. Ichihara, and E. Kusunose.** 1976. Superoxide dismutase from *Mycobacterium* species, strain Takeo. Arch. Microbiol. **108**:65–73.
15. **Lindgren, S., L. Enerback, and N. Freiberg.** 1976. Oral dermatitis acantholytic itching disease responding to dapsone. Dermatitis herpetiformis, pemphigus or a new disease. Oral Surg. Oral Med. Oral Pathol. **42**:597–605.
16. **Mandell, G. L., and E. W. Hook.** 1969. Leukocyte bactericidal activity in chronic granulomatous disease: correlation of bacterial hepdrogen peroxide production and susceptibility to intracellular killing. J. Bacteriol. **100**:531–532.
17. **McCord, J. M.** 1974. Free radicals and inflammation: protection of synovial fluid by superoxide dismutase. Science **185**:529–531.
18. **McCord, J. M., and I. Fridovich.** 1969. Superoxide dismutase. An enzymatic function for erythrocuprein (hemocuprein). J. Biol. Chem. **244**:6049–6055.
19. **McDougall, A. C., W. P. Horsfall, J. E. Hede, and A. J. Chaplin.** 1980. Splenic infarction and tissue accumulation of crystals associated with the use of clofazimine (Lamprene; B663) in the treatment of pyoderma gangrenosum. Br. J. Dermatol. **102**:227–230.
20. **Michaëlsson, G., L. Molin, S. Öhman, L. Gip, B. Lindström, M. Skogh, and I. Trolin.** 1976. Clofazimine. A new agent for the treatment of pyoderma gangrenosum. Arch. Dermatol. **122**:344–349.
21. **Molin, L.** 1975. Clofazimine-enhanced phagocytosis in pustulosis palmaris et plantaris. Acta Derm. Venereol. **55**:151–153.
22. **Nash, T.** 1953. The colorimetric estimation of formaldehyde by means of the Hahtzsch reaction. Biochem. J. **55**:416–422.
23. **Niwa, Y., S. Miyake, K. Ishimoto, M. Shingu, and M. M. Yokoyama.** 1982. Auto-oxidative damage in Behçet's disease—endothelial cell damage following the elevated oxygen radicals generated by stimulated neutrophils. Clin. Exp. Immunol. **49**:247–255.

23a. **Niwa, Y., T. Sakane, and Y. Miyachi.** 1984. Dissociation of the inhibitory effect of dapsone on the generation of oxygen intermediates in comparison with that of colchicine and various scavengers. Biochem. Pharmacol. **33**:2355–2360.

24. **Niwa, Y., T. Sakane, M. Shingu, and M. M. Yokoyama.** 1983. Effect of stimulated neutrophils from the synovial fluid of patients with rheumatoid arthritis on lymphocytes—a possible role of increased oxygen radicals generated by the neutrophils. J. Clin. Immunol. **3**:228–240.
25. **Ridley, D. S.** 1959. Bacterial indices, p. 371–372. *In* R. G. Cochrane (ed.), Leprosy in theory and practice, 1st ed. J. Wright & Sons Ltd., Bristol.
26. **Rojas-Espinosa, O.** 1978. Phagocytosis in leprosy. II. Production of superoxide by circulating blood leukocytes from lepromatous patients. Int. J. Lepr. **46**:341–377.
27. **Rosen, H., and S. J. Klebanoff.** 1979. Bactericidal activity of a superoxide anion-generating system. A model for the polymorphonuclear leukocyte. J. Exp. Med. **149**:27–39.
28. **Rosen, H., and S. J. Klebanoff.** 1981. Role of iron and ethylenediaminetetraacetic acid in the bactericidal activity of a superoxide anion-generating system. Biochem. Biophys. Res. Commun. **208**:512–519.
29. **Salin, M. L., and J. M. McCord.** 1975. Free radicals and inflammation. Protection of phagocytosing leukocytes by superoxide dismutase. J. Clin. Invest. **56**:1319–1323.
30. **Sher, R., R. Anderson, A. Glover, and A. A. Wadee.** 1978. Polymorphonuclear cell function in the various polar types of leprosy and erythema nodosum leprosum. Infect. Immun. **21**:959–965.
31. **Stach, J.-L., F. Fumoux, M. Strobel, A. Baret, and M. Michelson.** 1981. Immunologie-augmentation de l'activitié dismutasique dans les leucocytes spléniques de souris sensible au BCG et á mycobacterium lepraemurium. C.R. Acad. Sci. **293**:575–578.
32. **Stendahl, O., L. Molin, and C. Dahlgren.** 1978. The inhibition of polymorphonuclear leukocyte cytotoxicity by dapsone. J. Clin. Invest. **62**:214–220.
33. **Swanbeck, G., and G. Wennersten.** 1973. Effect of betacarotene on photohemolysis. Acta Derm. Venereol. **53**:283–289.
34. **Takigawa, M., Y. Miyachi, M. Uehara, and H. Tagami.** 1982. Treatment of *pustulosis palmaris et plantaris* with oral colchicine. Arch. Dermatol. **118**:458–460.
35. **Ward, P. A., S. Goralnick, and W. E. Bullock.** 1976. Defective leukotaxis in patients with lepromatous leprosy. J. Lab. Clin. Med. **87**:1025–1032.
36. **Winkelmann, R. K., and W. B. Ditto.** 1964. Cutaneous and visceral symptoms of necrotizing or "allergic" angitis: a study of thirty-eight cases. Medicine (Baltimore) **43**:59–89.
37. **Winkelmann, R. K., and H. L. Roth.** 1960. Dermatitis herpetiformis with acantholysis or pemphigus with response to sulfonamides. Arch. Dermatol. **82**:385–390.
38. **Winston, G. W., and A. I. Cederbaum.** 1983. NADPH-dependent production of oxygen radicals by purified components of the rat liver mixed function oxidase system. J. Biol. Chem. **258**:1508–1513.