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Effects of Clofazimine Alone or Combined with Dapsone on Neutrophil and Lymphocyte Functions in Normal Individuals and Patients with Lepromatous Leprosy

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The effects of clofazimine on neutrophil activities such as random motility, migration to the leukoattractants endotoxin-activated serum and *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine phagocytosis of *Candida albicans*, postphagocytic hexose-monophosphate shunt activity, and myeloperoxidase-mediated iodination and the effects of clofazimine on lymphocyte transformation to mitogens were assessed in vitro and after ingestion of the drug by normal individuals and patients with lepromatous leprosy. For in vitro studies, the concentration range of the drug investigated was 10^{-6} M to 10^{-2} M. For in vivo studies, subjects ingested 200 mg of clofazimine daily for a period of 5 days. At concentrations of 5×10^{-6} M to 5×10^{-3} M clofazimine caused a progressive dose-dependent inhibition of neutrophil motility without detectable effects on phagocytosis, postphagocytic hexose-monophosphate shunt activity, or myeloperoxidase-mediated iodination. Over the same concentration range, clofazimine inhibited lymphocyte transformation. The inhibitory effect on neutrophil motility was associated with a spontaneous stimulation of oxidative metabolism and could be prevented by coincubation of dapsone with clofazimine. After ingestion of clofazimine responsiveness of lymphocytes to mitogens was decreased in normal volunteers and leprosy patients; neutrophil motility in normal individuals was likewise inhibited.

Clofazimine (5), also known as B663 or Lamprene, is an extensively used anti-leprosy drug. Apart from its anti-mycobacterial activity, clofazimine has also been reported to possess anti-inflammatory activity (17). This property enhances the value of clofazimine in leprosy, since it may also be used to control adverse immunological reactions. In this study, we have investigated the effects of clofazimine in vitro and in vivo on the motility, phagocytosis, and postphagocytic metabolic activities of neutrophils and transformation to mitogens of lymphocytes from normal individuals and patients with lepromatous leprosy. The effects of combinations of clofazimine and dapsone on the motility of neutrophils from patients with leprosy have also been investigated.

MATERIALS AND METHODS

Chemotherapeutic agents. Clofazimine in 100-mg capsules of pure drug was obtained from Ciba-Geigy, Johannesburg. For in vitro studies, the drug was solubilized in dimethyl sulfoxide (DMSO) to give a concentration range of 10^{-6} M to 10^{-3} M (equivalent to 0.3 to 300 μg/ml) in Hanks balanced salt solution (HBSS) for studies of motility, 0.15 M phosphate-buffered saline for phagocytic studies and TC199 for lymphocyte studies. Both HBSS and TC199 were obtained from GIBCO Laboratories, Grand Island, N.Y. Control systems were included for each clofazimine concentration investigated, and these contained the corresponding DMSO concentrations: 0.0015 to 1.5% DMSO for 10^{-6} M to 10^{-3} M clofazimine. Dapsone, obtained from Lennon Ltd., Port Elizabeth, South Africa, was used at concentrations previously found to increase neutrophil motility in vitro (3).

Leprosy patients. We used 12 newly admitted untreated patients in this study (two patients with borderline-tuberculoid leprosy, two with borderline leprosy, six with borderline-lepromatous leprosy, and two with lepromatous leprosy). Only patients with borderline-lepromatous leprosy and lepromatous leprosy were used for the in vivo studies; this group comprised five patients with borderline-lepromatous leprosy and one patient with lepromatous leprosy. The group used for in vitro studies comprised two patients with borderline-tuberculoid leprosy, two with borderline leprosy, and two with lepromatous leprosy. The classification was based on the clinical and histopathological criteria of Ridley and Jopling (12).

For in vivo studies, six normal adult volunteers and six newly diagnosed patients with lepromatous leprosy ingested 200-mg capsules of clofazimine as one oral daily dose for 5 days. Tests of neutrophil motility and

lymphocyte transformation were performed before the ingestion of clofazimine, at 2 and 24 h after the ingestion of one oral dose of 200 mg of clofazimine, and on day 5 of ingestion of 200 mg of clofazimine daily (testing was performed 2 h after ingestion of the last dose).

Neutrophil motility. Neutrophils, obtained from heparinized venous blood (5 U of heparin per ml) from normal adult volunteers, were suspended in a final concentration of 3×10^6 to 5×10^6/ml in HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, Sigma Chemical Co., St. Louis, Mo.)-buffered HBSS after hypotonic lysis of residual erythrocytes with 0.84% ammonium chloride as previously described (4). The leukoattractants used were endotoxin-activated fresh autologous serum (EAS) and the synthetic leukotactic tripeptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (Miles Laboratories Inc., Elkhart, Ind.). Bacterial endotoxin (100 μg) (*Escherichia coli* O127:B8, Difco Laboratories, Detroit, Mich.) was added to 1 ml of serum, and the mixture was diluted eightfold with HBSS. *N*-Formyl-L-methionyl-L-leucyl-L-phenylalanine was used at a final concentration of 5×10^{-7} M in HBSS supplemented with 0.1% bovine serum albumin.

In random migration studies the leukoattractant was replaced with an equal volume of bovine serum albumin-supplemented HBSS. Assays of motility were performed in modified Boyden chambers by using 5-μm pore size membrane filters (Sartorius-membranfilter, Göttingen, West Germany) and a 2-h incubation period. Clofazimine together with the neutrophils was introduced into the upper compartment of the Boyden chamber. The combined effects of clofazimine and dapsone on neutrophil motility were investigated by using a constant concentration of 10^{-4} M clofazimine. Dapsone, which has previously been shown to stimulate neutrophil motility (3), was used at concentrations of 1×10^{-3} M, 2.5×10^{-3} M, and 5×10^{-3} M.

Phagocytic studies. Pure neutrophil suspensions in phosphate-buffered saline were prepared as previously described (4). Phagocytosis of *Candida albicans* was performed with a neutrophil/*C. albicans* ratio of 1:3 (4). Various concentrations of clofazimine were added in 0.1-ml volumes to give a final reaction volume of 1 ml. The results are expressed as the percentage of *C. albicans* ingested after incubation periods of 10 and 20 min.

HMS activity. Hexose-monophosphate shunt (HMS) activity was measured by a modified method of Wood et al. (4, 19). The reaction mixture of 1 ml contained 2×10^6 neutrophils, 0.1 ml of autologous serum, 10^7 *C. albicans*, 0.1 ml of the various clofazimine concentrations, and 0.06 μCi of D-[1-^{14}C]glucose (New England Nuclear Corp., Boston, Mass.). The effects of clofazimine on spontaneous HMS activity were investigated in systems using neutrophils, clofazimine, and [1-^{14}C]glucose only. The results are expressed as nanomoles of glucose metabolized per 2×10^6 neutrophils during a 60-min incubation period.

Chemiluminescence. To measure effects of clofazimine on spontaneous chemiluminescence 3×10^6 neutrophils were incubated with clofazimine (10^{-6} M to 10^{-3} M) in the presence of 10^{-3} M luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma Chemical Co.) in a reaction volume of 1 ml. Control systems contained no clofazimine. Tubes were incubated for 30 min at 37°C before the addition of luminol and then for 60 min more. Spontaneous chemiluminescence was measured at 10-min intervals on a Lumac Biocounter (model 2010, Lumac Systems Inc., Titusville, Fla.). The emitted light was measured and recorded as counts per minute.

Myeloperoxidase-mediated iodination of *C. albicans*. Myeloperoxidase-mediated iodination of *C. albicans* was determined by a modified method of Root and Stossel (4, 13). To 0.1 ml of neutrophil suspension (10^7/ml) were added 0.1 ml of *C. albicans* (10^8/ml), 0.1 ml of fresh autologous serum, 0.1 ml of the appropriate drug concentration, 0.1 ml of a ^{125}I-labeled solution (0.6 μCi/ml, sodium iodate, New England Nuclear Corp.) and 0.5 ml of HBSS. After incubation at 37°C for 60 min the incorporation of ^{125}I into acid-precipitable protein was determined by solid scintillation counting. The results are expressed as nanomoles of ^{125}I contained in the protein precipitate.

Lymphocyte studies. The in vitro and in vivo effects of clofazimine on lymphocyte transformation to the mitogens phytohemagglutinin and concanavalin A at concentrations of 25 and 50 μg/ml for both mitogens were also determined. Mononuclear cells obtained by density gradient centrifugation were washed and suspended in TC199 to a concentration of 4×10^6/ml. Lymphocytes, 2×10^5 in 50 μl, were placed in the wells of 6-mm Linbro tissue culture plates (Flow Laboratories, Inc., Rockville, Md.) and brought to 0.2 ml by the addition of serum-supplemented TC199 (containing 20% autologous serum), 20 μl of the appropriate drug concentration, and 20 μl of each mitogen. The plates were incubated for 48 h at 37°C in a humidified atmosphere of air and 3% CO_2, after which 0.2 μCi of tritiated thymidine (New England Nuclear Corp.) in 20 μl was added to each well, and the plates were incubated for 18 h more. The plates were harvested with a multiple automated sample harvester, and the extent of incorporation of tritiated thymidine into newly synthesized DNA was assessed in a liquid scintillation spectrophotometer.

RESULTS

The results are expressed as mean values ± standard error of four to six separate experiments. Statistical analyses of results were done by Student's *t* test.

Neutrophil motility. Clofazimine caused a dose-dependent inhibition of neutrophil migration in vitro. This was observed with neutrophils obtained from normal individuals and patients with leprosy. Results for EAS are shown in Table 1. Neutrophil motility was unaffected by DMSO concentrations of <1.5% (results not shown). Ingestion of clofazimine by normal individuals was associated with decreased neutrophil migration. This inhibitory effect was evident 2 h after the ingestion of one oral dose of clofazimine and on day 5 of ingestion of clofazimine (200 mg) daily. These results, which were not statistically significant, are shown in Table 2. No change in neutrophil migration was observed after ingestion of clofazimine by leprosy patients (results not shown).

TABLE 1. In vitro effects of clofazimine on neutrophil motility from normal individuals and patients with leprosy to autologous EAS

Test system	Migration to EAS of neutrophils from:	
	Normal subjects[a]	Patients with leprosy[b]
Control (no clofazimine)	143 ± 26	76 ± 26
10^{-3} M clofazimine	35 ± 10[c]	11 ± 7[c]
10^{-4} M clofazimine	70 ± 13[c]	36 ± 14[d]
10^{-5} M clofazimine	121 ± 23[d]	46 ± 20

[a] Values are means ± standard error for six people, expressed as cells per high-powered microscope field.
[b] Values are means ± standard error for five people, expressed as cells per high-powered microscope field.
[c] $P < 0.01$.
[d] $P < 0.05$.

Coincubation of 10^{-4} M clofazimine with dapsone in vitro. Coincubation of 10^{-4} M clofazimine with dapsone in vitro eliminated the clofazimine-mediated inhibition of migration to EAS. Dapsone, as previously reported (3), stimulated neutrophil motility (Table 3).

Phagocytic studies. Clofazimine at concentrations of 10^{-6} M to 10^{-4} M had no effects on phagocytosis of *C. albicans* by neutrophils. Greater concentrations of clofazimine caused inhibition of phagocytosis and postphagocytic HMS activity and myeloperoxidase-mediated iodination of *C. albicans*. However, these effects were due entirely to the high concentrations of DMSO (1.5%). Clofazimine at concentrations of $>10^{-6}$ M increased spontaneous (resting) HMS activity and chemiluminescence. Results for 10^{-4} M clofazimine are shown in Table 4.

Lymphocyte transformation. The responsiveness of lymphocytes from normal individuals to mitogens was significantly decreased by clofazimine at concentrations of $>10^{-5}$ M. The control value for 25 μg of phytohemagglutinin per ml was 57,841 ± 9,775 radioactive cpm, and this decreased to 30,536 ± 7,064 in the presence of 10^{-4} M clofazimine. This difference was statistically significant ($P < 0.05$). DMSO caused inhibition of lymphocyte transformation only at concentrations of >0.15%. The inhibition of transformation caused by clofazimine at concentrations of 10^{-5} M to 10^{-4} M is therefore a true effect. Ingestion of clofazimine by normal individuals was associated with inhibition of lymphocyte transformation to both mitogens at both concentrations used. The inhibition was observed 2 h after ingestion of one 200-mg oral dose of clofazimine (with a return to almost normal values after 24 h) and on day 5 of daily ingestion of clofazimine. These results are shown in Table 2. Similar but less striking effects were observed in patients with leprosy after ingestion of clofazimine (Table 5).

DISCUSSION

Clofazimine at concentrations of $>10^{-6}$ M has been found to inhibit neutrophil motility and lymphocyte transformation in vitro. These activities of the drug may be related to its reported anti-inflammatory properties (9, 11, 14, 17). Clofazimine, which increases phagocytosis by mac-

TABLE 2. Effects of ingestion of clofazimine by normal individuals on neutrophil random motility, migration to EAS, and *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine and lymphocyte transformation to phytohemagglutinin and concanavalin A

Time of testing	Random motility[a]	Migration to[a]:		Lymphocyte transformation to[b]:	
		EAS	f-met-leu-phe	PHA (25 μg/ml)	ConA (25 μg/ml)
Before ingestion of clofazimine	34 ± 13[a]	192 ± 29	114 ± 21	59,340 ± 5,233	6,968 ± 1,119
2 h after ingestion of one oral dose of clofazimine (200 mg)	22 ± 7	117 ± 41	100 ± 10	28,021 ± 3,035	3,237 ± 446[c]
24 h after ingestion of one oral dose of clofazimine (200 mg)	28 ± 5	135 ± 15	120 ± 12	55,508 ± 3,245	4,501 ± 560[c]
On day 5 of ingestion of 200 mg of clofazimine daily	19 ± 7	165 ± 34	65 ± 15	45,985 ± 3,603[c]	4,113 ± 474[c]

[a] Results are means ± standard error for six people, expressed as the number of cells per high-powered microscope field. f-met-leu-phe, *N*-Formyl-L-methionyl-L-leucyl-L-phenylalanine.
[b] Results are means ± standard error for six people, expressed as radioactive counts per minute. PHA, phytohemagglutinin; ConA, concanavalin A.
[c] $P < 0.05$.

TABLE 3. Effects of coincubation of 10^{-4} M clofazimine with migration-stimulatory concentrations of dapsone on neutrophil migration from leprosy patients to autologous EAS

Test system	Migratory responsiveness to EAS[a]
Neutrophils + HBSS (control)	76 ± 26
Neutrophils + 10^{-4} M clofazimine	36 ± 14[b]
Neutrophils + 10^{-4} M clofazimine + 1 × 10^{-3} M dapsone	131 ± 40[c]
Neutrophils + 10^{-4} M clofazimine + 2.5 × 10^{-3} M dapsone	174 ± 42[c]
Neutrophils + 10^{-4} M clofazimine + 5 × 10^{-3} M dapsone	182 ± 64

[a] Results are means ± standard error of five experiments, expressed as the number of neutrophils per high-powered microscope field.
[b] $P < 0.05$ for inhibition of migration.
[c] $P < 0.05$ for stimulation of migration.

rophages (6), had no effects on neutrophil phagocytosis, postphagocytic HMS activity, and myeloperoxidase-mediated iodination of *C. albicans*. However clofazimine did cause increased spontaneous HMS activity and chemiluminescence by neutrophils in vitro. Similar effects of the drug on macrophage oxidative metabolism have been described (8). DMSO at high concentrations interfered with the assays of neutrophil and lymphocyte functions. The solvent stimulated neutrophil migration and inhibited neutrophil phagocytosis, HMS activity and myeloperoxidase-mediated iodination at concentrations of 1.5% and greater. These effects may be related to the anti-oxidant properties of DMSO (3).

Ingestion of 200 mg of clofazimine daily, which gives peak serum levels of 0.7 to 1 μg/ml (20), was associated with decreased neutrophil motility and lymphocyte transformation to mitogens in normal volunteers. The inhibition of random motility and migration to both leukoattractants was not statistically significant. However, since decreased motility was consistently observed, we believe it is a true effect of the drug. Ingestion of clofazimine by patients with leprosy was also accompanied by decreased lymphocyte responsiveness to mitogens. However there were no detectable effects on neutrophil motility, which may indicate that the major anti-inflammatory activity of the drug is to inhibit T-lymphocyte reactivity. Alternatively, prolonged intake of clofazimine may be necessary to demonstrate significant depression of neutrophil migration.

The ability of clofazimine to increase spontaneous oxidative metabolism may be related to inhibition of neutrophil motility. It has previously been reported that oxidation of neutrophils and lymphocytes reduces their respective migratory and blastogenic responses (16). Clofazimine by enhancing spontaneous oxidative metabolism may auto-oxidize leukocytes with consequent inhibition of cell locomotion and proliferation. This possibility is supported by the finding that the anti-oxidant dapsone completely blocked the clofazimine-mediated inhibition of neutrophil motility in vitro. However, this antagonistic effect of the two drugs on neutrophil motility may occur as a result of inactivation of clofazimine by dapsone. Although the dapsone concentrations used in these studies are higher than those attainable in vivo, we have previously found that similar effects occur in vivo after ingestion of 100 mg of dapsone (3).

The immunosuppressive properties of clofazimine are not shared by dapsone which is also a widely used anti-leprosy drug. Dapsone has been reported to cause increased neutrophil migration in vitro and in vivo and lymphocyte proliferation in vivo (3). Neutrophil motility is markedly decreased in untreated individuals

TABLE 4. In vitro effects of 10^{-4} M clofazimine on spontaneous HMS activity and chemiluminescence of blood neutrophils

Test system	HMS activity[a]	Chemiluminescence[b]
Control (no clofazimine)	2.4 ± 0.7	5,584 ± 902
10^{-4} M clofazimine	4.1 ± 0.4	9,404 ± 286

[a] Results are means ± standard error of four experiments, expressed as nanomoles of glucose metabolized.
[b] Results are means ± standard error of four experiments, expressed as counts per minute.

TABLE 5. Effects of ingestion of clofazimine by patients with leprosy on lymphocyte transformation to phytohemagglutinin and concanavalin A

Time of testing	Lymphocyte transformation to[a]: PHA (25 μg/ml)	ConA (25 μg/ml)
Before ingestion of clofazimine	59,814 ± 781	5,283 ± 1,779
2 h after ingestion of one oral dose of clofazimine (200 mg)	63,838 ± 1,101	5,354 ± 1,301
On day 7 of daily ingestion of clofazimine (200 mg)	46,794 ± 5,471	1,512 ± 456[b]

[a] Values are means ± standard error for six people, expressed as radioactive counts per minute. PHA, Phytohemagglutinin; ConA, concanavalin A.
[b] $P < 0.01$.

with lepromatous leprosy (7, 15, 18) and improves with dapsone therapy as does lymphocyte transformation (1, 2). In patients with leprosy, antimicrobial therapy with dapsone may improve cellular immunity with an increased probability of occurrence of adverse immunological reactions. Therapy with clofazimine, which inhibits cellular immune functions, should be accompanied by fewer such reactions and may be useful in providing good antimicrobial therapy together with a measure of control of harmful immunological reactions. Studies on the combined effects of clofazimine and dapsone on neutrophil migration in vitro suggest that clapsone may nullify the anti-inflammatory effects of clofazimine. Combinations of dapsone and clofazimine may therefore not be compatible in the chemotherapy of leprosy patients with adverse immunological reactions such as erythema nodosum leprosum. This possibility should be investigated in patients with erythema nodosum leprosum, but must not be confused with the beneficial effects of clofazimine-dapsone combinations for leprosy caused by drug-resistant *Mycobacterium leprae*.

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