The *in vitro* and *in vivo* effects of clofazimine on the motility of neutrophils and transformation of lymphocytes from normal individuals

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Summary The effects of clofazimine on neutrophil motility to endotoxin-activated serum and mitogen-induced lymphocyte transformation of leucocytes from normal individuals *in vitro*, and after ingestion of clofazimine by normal adult volunteers have been assessed. Clofazimine caused a progressive dose-dependent inhibition of neutrophil motility and of lymphocyte transformation *in vitro*. Ingestion of the drug by normal volunteers was accompanied by decreased neutrophil motility and lymphocyte transformation to mitogens. These findings suggest that the anti-inflammatory properties of clofazimine are related to inhibition of these cellular immune functions.

Introduction

The antimicrobial therapy of leprosy is more complicated than that of other acute or chronic bacterial infections. This is due to the development of adverse immunological reactions which may accompany chemotherapy. The conditions associated with these reactions are referred to as erythema nodosum leprosum (ENL) and the reversal reaction caused by types III and IV immunological hypersensitivity reactions respectively. These complications probably develop

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due to two related factors: (a) the extremely high antigen load found in patients with the lepromatous form of the disease and (b) the high degree of disease-associated immunosuppression, i.e. specific anergy to lepromin¹ and decreased neutrophil motility.² Drug-induced ENL is probably due to the destruction of leprosy bacilli with the formation of localized and circulating immune complexes and the development of tissue damage mediated by the phagocyte-complement system. Likewise, the decreased antigen load which accompanies successful antimicrobial therapy may result in a decrease in the specific unresponsiveness to the leprosy antigens with activation of hitherto suppressed antigen-specific T-lymphocytes. This may cause increased macrophage recruitment with resultant tissue damage due to cell-mediated mechanisms. These complications could develop solely as a consequence of the antimicrobial activity of drugs used in patients with leprosy.

However, a second more direct mechanism of induction of chemotherapy-associated hypersensitivity reactions may occur if the chemotherapeutic agents *per se* possess immunostimulatory activity as has previously been reported for dapsone.^{3,4} This agent was found to cause stimulation of neutrophil motility and a possible role for this drug in the pathogenesis of some cases of ENL suggested.³ There are reports that clofazimine is useful in the treatment of some cases of ENL^{5,6} and in controlling some reversal reactions.^{6,7} It has been shown in animal studies that clofazimine may possess anti-inflammatory properties⁸ although the precise mechanisms of this are unknown.

In this study we have investigated the effects of clofazimine on neutrophil motility and lymphocyte transformation in normal individuals *in vitro* and *in vivo*.

Materials and methods

Clofazimine (B663, lamprene) pure substance for *in vitro* studies and 100 mg capsules for *in vivo* studies was kindly donated by Ciba-Geigy (Pty) Ltd, Johannesburg. For *in vitro* studies the drug was solubilized in dimethylsulphoxide (DMSO) to give a concentration range of 10^{-6} M to 10^{-3} M (equivalent to 0.3 to 300 μ g/ml) in Hanks' balanced salt solution (HBSS) for studies of motility, and TC199 for lymphocyte studies. (Both HBSS and TC199 were obtained from the Grand Island Biological Co., Grand Island, NY, USA). Control systems containing the corresponding DMSO dilution only were included for each clofazimine concentration tested, i.e. 0.0015 to 1.5% DMSO for 10^{-6} M to 10^{-3} M clofazimine. For *in vivo* studies 6 normal adult volunteers ingested 2×100 mg capsules of clofazimine as a single oral, daily dose for 5 days. Tests of neutrophil motility and lymphocyte transformation were performed prior to the ingestion of clofazimine, at 2 h and at 24 h after ingestion of a single oral dose of 200 mg clofazimine and on the 5th day of ingestion of 200 mg of clofazimine daily (testing was performed 2 h after ingestion of the last dose).

Neutrophil motility. Neutrophils were obtained from heparinized blood (5 units of heparin/ml) from normal adult volunteers and were resuspended to a final concentration of 3×10^6 /ml in HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid obtained from the Sigma Chemical Co., St. Louis, Mo., USA) buffered HBSS following hypotonic lysis of residual erythrocytes with 0.84% ammonium chloride as previously described.⁹ The leucoattractant used was endotoxin-activated serum (EAS). Fresh autologous serum was activated with 100 μ g bacterial endotoxin per ml (*Escherichia coli* 0127:B8, Difco Laboratories, Detroit, Mich., USA) and diluted 8-fold with HBSS before use. The assays of motility were performed in modified Boyden chambers¹⁰ using 5 μ m-pore size membrane filters (Sartorius-membranfilter, Göttingen, West Germany) and a 2-h incubation period. The results are expressed as the number of cells which have completely crossed the filter, per microscope high-powered field as an average of triplicate filters.

Lymphocyte transformation. Blood for studies of lymphocyte function was defibrinated and fractionated by density gradient centrifugation (Ficoll-sodium metrizoate) at 400 g for 25 min. The mononuclear cells were washed twice in TC199 and resuspended to 4×10^6 /ml in TC199 supplemented with 20% autologous serum; 2×10^5 cells were used in the assay system which was done as previously described using phytohaemagglutinin (PHA) and concanavalin A (Con A) as the mitogens at concentrations of 25 and 50 μ g/ml.⁹

Results

The results are expressed as the mean value with standard error for 6 separate experiments for *in vitro* studies and 6 different individuals for *in vivo* studies. Statistical analyses were performed by Student's t-test for paired means.

Neutrophil motility. Clofazimine at concentrations greater than 5×10^{-6} M *in vitro* caused a progressive inhibition of neutrophil motility to EAS. These results are shown in Table 1. DMSO at all concentrations used had no significant effect on motility to EAS (results not shown). No effect on neutrophil motility to EAS was observed 2 h after ingestion of clofazimine by the group of normal individuals. However, a consistent slight inhibition of motility was observed 24 h after the ingestion of a single oral 200 mg dose of clofazimine and on the 5th day of clofazimine ingestion. This difference did not achieve statistical significance. These results are shown in Table 2.

Lymphocyte transformation. Incubation of lymphocytes with clofazimine at concentrations greater than 10^{-5} M *in vitro* caused inhibition of lymphocyte responsiveness to both mitogens at both concentrations (Table 1). DMSO, at a concentration of 1.5%, contained in the 10^{-3} M clofazimine system, caused inhibition of transformation. Lower concentrations had no detectable effects on the assay system. Ingestion of clofazimine was accompanied by a

Table 1. The effects of clofazimine on neutrophil motility to EAS and lymphocyte transformation to PHA and Con A *in vitro*

Clofazimine concentration	Neutrophil motility to EAS	Lymphocyte transformation to	
		PHA (25 µg/ml)	Con A (50 µg/ml)
Control	143 ± 26†	57,841 ± 9,775‡	8,830 ± 4,767‡
10 ⁻³ M	35 ± 10**	N.S.§	N.S.§
10 ⁻⁴ M	70 ± 13**	30,536 ± 7,063*	1,764 ± 696*
10 ⁻⁵ M	121 ± 23*	59,278 ± 11,817	7,497 ± 3,583

†Results as mean neutrophils/microscope high-powered field with standard error for 6 experiments.

‡Results as mean radioactive counts per minute with standard error for 3 experiments.

§Results not shown due to the inhibitory effects of DMSO on the reaction system.

**P* < 0.05.

***P* < 0.01.

Table 2. The effects of ingestion of clofazimine by 6 normal adult volunteers on neutrophil motility to EAS and lymphocyte transformation to PHA and Con A

Time of testing	Neutrophil motility to EAS	Lymphocyte transformation to	
		PHA (25 µg/ml)	Con A (25 µg/ml)
1 Before ingestion of clofazimine	192 ± 29†	59,339 ± 5,233‡	6,968 ± 1,119
2 2 h after the ingestion of a single oral dose of 200 mg clofazimine	177 ± 41	28,021 ± 3,305*	3,237 ± 446**
3 24 h after ingestion of a single oral dose of 200 mg clofazimine	135 ± 35	55,508 ± 3,245	4,502 ± 560
4 On the 5th day of ingestion of 200 mg of clofazimine daily	165 ± 34	45,985 ± 3,603*	4,127 ± 474*

†Results as mean neutrophils per microscope high-powered field with standard error for 6 individuals.

‡Results as mean radioactive counts per minute with standard error for 6 individuals.

**P* < 0.05.

***P* < 0.01.

decreased lymphocyte response to both mitogens in each individual tested. The inhibitory effects on transformation were detected 2 h after the ingestion of a single 200 mg oral dose of clofazimine and on the 5th day of ingestion of clofazimine. The lymphocyte transformation to PHA returned to normal values 24 h after the ingestion of a single oral dose of 200 mg clofazimine. These results are shown in Table 2.

Discussion

In this study we have found that clofazimine causes inhibition of neutrophil motility to EAS and of lymphocyte proliferation to mitogens *in vitro* and *in vivo*. Although the observed changes in neutrophil motility *in vitro* following exposure to clofazimine *in vivo* were not significant, they were consistently found and we believe that this is a true effect of clofazimine. These findings support to some extent previous reports that clofazimine may be of value in the treatment of ENL^{5,6} and reversal reactions.^{6,7} However, this does not exclude the possibility that in some cases clofazimine may precipitate these reactions by virtue of its ability to cause antigen release through antimicrobial mechanisms. Nevertheless, as a result of its immuno-inhibitory activity clofazimine therapy should be accompanied by considerably fewer adverse immunological reactions than dapsone, which possesses immunostimulatory activity. Clofazimine should therefore be considered to be a useful agent in the antimicrobial therapy of those patients undergoing serious immunological reactions or those who may be prone to developing such reactions since it will not counteract the therapeutic activity of prescribed anti-inflammatory or immunosuppressive agents. Indeed it could be expected to enhance their activity.

In describing the immunological effects of clofazimine we prefer to use the term immuno-inhibitory rather than anti-inflammatory. The use of the term anti-inflammatory is often taken as being synonymous with immunosuppressive. However, we feel that this implication is not necessarily correct. Indeed dapsone, which is used in a variety of dermatological conditions as an 'anti-inflammatory' agent,¹¹ has been found to possess non-specific immunostimulatory activity.⁴ It is therefore possible that in some cases anti-inflammatory agents may possess immunostimulatory activity. This concept seems difficult to reconcile. Nevertheless, it is possible that dapsone by virtue of its ability to protect leucocyte membranes from auto-oxidation which causes marked inhibition of locomotion⁴ may permit leucocytes, especially neutrophils, to enter inflammatory zones, phagocytose immune complexes and leave these zones. However, if they enter the zones and undergo strong auto-oxidation during function they may become immobilized and further contribute to tissue damage and chronic inflammation by release of proteolytic enzymes and toxic oxygen radicals.¹² This theory could rationalize the anti-inflammatory activity of dapsone in some conditions and its possible pro-inflammatory activity in ENL in which it may enhance the phagocyte response to chronic antigen release.

Clofazimine, as shown in this study, has the opposite effect to dapsone, i.e. it causes inhibition of neutrophil motility and lymphocyte transformation. These findings may explain the mechanism by which clofazimine is of value in the treatment of some ENL and reversal immunity reactions.

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