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# The action of Clofazimine on the level of lysosomal enzymes of cultured macrophages

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## SUMMARY

Mouse peritoneal and calf alveolar macrophage cultures were exposed to various concentrations of Clofazimine, 3 (*p*-chloroanilino)-10-p-chlorophenyl 2, 10-dihydro-2-isopropylimino, for 120 hr and an increase of four lysosomal enzymes were found with 0·3 μg/ml of the drug. In mouse peritoneal macrophage cultures, higher concentrations were toxic. Cycloheximide inhibited the lysosomal enzyme activity increase found. No change in enzymatic activity was observed when a lysosomal enriched granular fraction was incubated with various drug concentrations. Our results strongly suggest that Clofazimine at concentrations close to therapeutic serum levels induces *de novo* synthesis of lysosomal enzymes in macrophage cultures.

## INTRODUCTION

The mode of action of Clofazimine, 3 (*p*-chloro anilino)-10-p-chlorophenyl 2, 10-dihydro-2-isopropylimino, an anti-leprotic drug, remains elusive. There is experimental evidence suggesting that Clofazimine modifies the function of the lysosomal apparatus. Brandt has reported an enhancing effect of the drug on the phagocytic capacity of neutrophilic leucocytes (Brandt, 1971) and macrophages (Brandt & Svensson, 1973) and similar observations have been published by others (Michaëlsen *et al.*, 1976; Molin, 1975). Cline demonstrated that Clofazimine potentiated the killing of *Listeria monocytogenes* by macrophages (Cline, 1970). The increased anti-listeria activity by activated macrophages has been well documented (North, 1974). There are morphological, functional and biochemical differences between activated and resting macrophages (Karnovsky *et al.*, 1975). Activated cells are well spread, showing increased membrane ruffling, phagocytic ability and lysosomal enzyme content (Danneberg *et al.*, 1974; Mackaness, 1970).

The above mentioned observations prompted us to study the action of Clofazimine on the lysosomal enzyme content of cultured macrophages. We thought that its beneficial effect on leprosy could be, at least, partially explained, through an action of the drug on the macrophage lysosomal apparatus.

## MATERIALS AND METHODS

*Experimental animals.* Swiss mice were obtained from the mouse colony established at the National Institute for Scientific Research (CENIC), Havana, Cuba. Alveolar macrophages were obtained aseptically from healthy calves from a local slaughtering house.

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*Biochemical reagents.* Phenolphthalein glucuronic acid 0·01 M, pH 7·0, leucine–2–naphthylamide, glycyl phenylanine naphthylamide, *N*–benzoyl–DL–arginine–naphthylamide HCl (BANA), Fast Garnett GBC, bovine serum albumin and cycloheximide were from Sigma Chemical Co., UK, p–nitrophenyl–β–D–galactopyranoside and *p*–nitrophenyl–2–acetamido–β–D–deoxyglucopyranoside from Koch Light, UK. Highly polymerized calf thymus DNA from Drug House, UK, diamino benzoic acid from Aldrich Biochemical, USA. Clofazimine was a gift from Ciba Geigy Ltd., Basle, Switzerland.

*Macrophage collection and culture.* Macrophages were obtained essentially as previously described (Finlay, Davies & Allison, 1975). Briefly macrophages were obtained by peritoneal lavage of mice with M-199 (Burroughs Wellcome, UK) containing 100 U/ml of penicillin and 100 μg/ml streptomycin and 10 U/ml heparin (preservative free, Boots, UK) without serum. Five ml aliquots of the collected fluid containing $0{\cdot}6 \times 10^6$ cells/ml were distributed into 50 mm plastic petri dishes (culture grade, Nunc, Denmark) and incubated in a humidified atmosphere of 5% carbon dioxide and air at 37°C overnight to allow attachment of adherent cells. Non-adherent cells were removed by washing four times with sterile saline. Morphologically more than 90% of the adherent cell population were macrophages and 98% viable by trypan blue exclusion. The adherent cells were then cultured in M-199 containing 30% of heat inactivated newborn calf serum. Batches of newborn calf serum used in the experiments were collected at the CENIC animal colony. Calf alveolar macrophages were obtained following the method described by Myrvik, Leake & Fariss (1961) for rabbits. Alveolar cells were plated at $0{\cdot}5 \times 10^6$ cells/ml. After allowing cells to attach overnight the plates were washed and cultured as indicated above. Solutions of Clofazimine were prepared from a concentrated stock solution of 10 mg of Clofazimine dissolved in 1 ml of dimethyl sulphoxide (DMSO).

The concentrated Clofazimine solution was diluted with serum and M-199 was added, since the drug was not soluble if added directly to the culture medium containing serum in the concentration to be used. The final concentration of DMSO was always 0·1% v/v. Control cultures contained only DMSO. Clofazimine was added to the cultures immediately after washing with sterile saline and left in contact with the cells for up to 120 hr.

The experiment was ended by removing the medium and washing the cell layer once with saline. The cells were then released by adding saline containing 0·1% v/v Triton X-100 and scraping with sterile silicon rubber bungs. Enzyme activity was assayed in the solution containing disrupted cells.

*Preparation of granular fraction from calf alveolar macrophages.* Cells were obtained following the method of Myrvik *et al.* (1961). Alveolar cells were washed three times with sterile saline. More than 90% were macrophages. Cells were resuspended in cold 0·25 M sucrose-imidazole 3 mM (pH 7·4) and ruptured with a Dounce homogenizer. Nuclei and intact cells were sedimented by centrifugation at 5,300 $g$ $min^{-1}$. The supernatant was centrifuged at 222,000 $g$ $min^{-1}$. The pellet was resuspended in the same solution supplemented with Triton X-100, final concentration 0·1% v/v and this was used as the granular fraction for lysosomal enzyme assays.

*Enzyme assays.* All assays were performed under conditions giving linear release of product in relation to the amount of sample used and the time of incubation.

β–glucuronidase was assayed by the method of Talalay, Fishman & Huggins (1946). β–galactosidase was assayed by the method of Conchie, Findlay & Levy (1959) using *p*–nitrophenil–β–D–galactopyranoside as substrate. *N*–acetyl–β–D–glucosaminidase was assayed by the method of Wollen, Heyworth & Walker (1961), using *p*–nitrophenyl–2–acetamido–2–β–D–glucopyranoside as substrate dissolved in 0·1 M citrate buffer pH 4·5. Catepsin B was assayed by the method of Barrett (1972). Catepsin C was assayed as suggested by Barrett (1972) and developed by Finlay *et al.* (1975).

Leucine–2–naphthylamidase was assayed by the method of Goldbarg and Rutenberg (1958) as modified by Davies, Krakauer and Weissmann (1970).

*Protein assay.* The method of Lowry *et al.* (1951) was employed using bovine serum albumin as standard.

*DNA assay.* The fluorimetric method of Kissane & Robbins (1958) was employed using highly polymerized calf thymus DNA as standard.

*Morphological observations.* Cultures were observed daily by means of an Olympus inverted stage microscope.

*Statistical tests.* Means and standard deviations were calculated after samples were shown to be homogenous by calculation of coefficient of variance. Significance of difference was established by the Student's *t*-test. In all experiments quadruplicate cultures were done and biochemical data are expressed as mean ± 1 s.d. Each experimental series described in this paper was repeated at least three times with the same results.

## RESULTS

*Biochemical changes in macrophage cultures exposed to Clofazimine for 120 hr*

Peritoneal mouse macrophage cultures were exposed to a wide dose range of Clofazimine varying from 0·003 to 9 μg/ml. Exposure of macrophages to 0·3 μg/ml resulted in a significant increase in their lysosomal enzyme activity, more pronounced in cultures exposed to the higher drug concentration (Table 1). Four enzymes, three glycosidases and one protease were assayed with similar results. Exposure to 0·03 μg/ml showed a significant increase in just two enzymes (Cathepsin B and β–glucuronidase) while at the 0·3 μg/ml the increase appeared in all enzymes. Protein concentrations were similar in cultures treated in the range of 0 to 0·3 μg/ml inclusive. Higher concentrations of Clofazimine (3 and 9 μg) showed a significant decrease, indicating cell detachment. Larger, better spread cells were found in cultures exposed to 0·3 μg/ml at 0·03 μg/ml similar but less evident changes were present.

In order to determine if Clofazimine provoked the same behaviour in macrophages of a different species and source, we cultured calf alveolar macrophages for the same period of time (120 hr) in the presence of varying concentrations of Clofazimine (0·03–6 μg/ml). At the end of the culture period, cells exposed to drug concentrations higher than 0·3 μg/ml were larger and more granular than control cultures. No signs of morphological toxicity appeared at higher drug concentrations. An evident change was in the colour of the cells which became bright yellow gradually as the drug concentration increased. Two lysosomal enzymes, *N*–Acetyl–β–D–glucosaminidase and β–galactosidase, were biochemically assayed. Cellular levels of both enzymes increased significantly in cultures exposed to 0·3 μg/ml or higher. No significant change in protein content was observed (Table 2).

*Time dependent increase of lysosomal enzyme levels*

Mouse peritoneal macrophages were exposed to concentrations 0·3 and 6 μg/ml of Clofazimine for various periods of time up to 120 hr. Every 24 hr morphological observations and biochemical assays were performed. Control cultures behaved as reported by Cohn & Benson (1965), showing a

**Table 1.** Lysosomal enzyme levels and protein content in mouse peritoneal macrophages exposed to various concentrations of Clofazimine for 120 hr

| Concentration of Clofazimine μg/ml | nmol product formed by substrate hydrolysis/mg of protein/hr | | | | μg/plate |
|---|---|---|---|---|---|
| | *N*–acetyl–β–D–glucosaminidase | Cathepsin B | β–glucuronidase | β–galactosidase | protein |
| 0 | 1632 ± 148 | 122 ± 10·3 | 68 ± 5·2 | 92 ± 4·6 | 570·7 ± 73·4 |
| 0·003 | 1786 ± 192 | 139 ± 11·4 | 76 ± 4·4 | 100 ± 6·7 | 548·9 ± 56·3 |
| 0·03 | 1796 ± 175 | 143 ± 8·9* | 78 ± 5·6* | 100 ± 7·5 | 533·2 ± 33·8 |
| 0·3 | 2694 ± 201‡ | 165 ± 12·1‡ | 99 ± 7·8‡ | 149 ± 9·2‡ | 550·9 ± 90·5 |
| 3 | 1382 ± 153 | 49 ± 9·3‡ | 43 ± 6·4‡ | 79 ± 3·8† | 386·4 ± 26·6* |
| 9 | 938 ± 169‡ | 43 ± 8·6‡ | 22 ± 5·7‡ | 31 ± 8·9‡ | 240·2 ± 14·9‡ |

* $P < 0·05$; † $P < 0·01$; ‡ $P < 0·001$.

**Table 2.** Lysosomal enzyme levels and protein content in alveolar macrophages exposed to various concentrations of Clofazimine for 120 hr

| Concentration of Clofazimine (μg/ml) | nmol product released by substrate hydrolysis/mg of protein/hr | | μg/plate |
|---|---|---|---|
| | N-Acetyl β-D-glucosaminidase | β-galactosidase | protein |
| 0 | 2188±218 | 125±14·8 | 1024·6±101·3 |
| 0·03 | 2615±225* | 153±13·5* | 1068·3±117·7 |
| 0·3 | 2680±194* | 152±11·4* | 1136·2±126·5 |
| 3 | 2870±237† | 177±15·9† | 1098·4± 90·4 |
| 6 | 2707±210† | 168±12·6† | 1102·2± 86·4 |

* $P<0·05$; † $P<0·01$.

steady increase of the lysosomal enzyme level which correlated with morphological changes expressed as an increase in cell size and granularity. After 24 hr, the level of lysosomal enzymes was significantly greater in cell cultures treated with the drug concentration of 0·3 μg/ml than in controls (Table 2). However, after 48 hr, cells treated with the drug concentration of 6 μg/ml expressed a decrease of the lysosomal enzyme content as compared to control cultures. The morphological changes observed in the previous set of experiments at these concentrations appeared after 48 hr and became more pronounced as time passed.

Leucine–2–naphthylamidase, a membrane associated enzyme, was also biochemically assayed. An increased activity was only found in cell cultures treated with 0·3 μg/ml at 96 and 120 hrs. This strongly suggests a different behaviour of this enzyme as compared to the lysosomal enzymes studied.

24 hr cultures showed similar DNA content while 120 hr cultures showed a significant decrease in the 6 μg/ml concentration, indicating a decline in cell number.

**Table 3.** The time dependent effect of Clofazimine treatment on cellular enzyme levels in mouse peritoneal macrophages

| Enzyme | concentration of Clofazimine (μg/ml) | Time (hr) 0 | 24 | 48 | 72 | 96 | 120 |
|---|---|---|---|---|---|---|---|
| β-galactosidase | 0 | 2·0±0·4 | 2·35±0·3 | 3·14±0·2 | 4·13±0·3 | 4·41±0·6 | 4·90±0·5 |
| | 0·3 | 1·9±0·3 | 2·98±0·1† | 3·50±0·1* | 5·04±0·2 | 5·8±0·5* | 6·31±0·2† |
| | 6 | 1·9±0·4 | 2·80±0·1* | 3·59±0·2* | 3·7±0·4 | 3·69±0·4 | 3·24±0·3† |
| CathepsinC | 0 | 94·8±10·6 | 114±0·6 | 192±15·7 | 201±11·6 | 204±18·6 | 218±16·4 |
| | 0·3 | 97·5±12·8 | 158±8·5‡ | 229±12·5* | 234±14·7* | 252±21·3* | 272±14·9† |
| | 6 | 90·9±11·2 | 123±7·8 | 173±17·9 | 184±12·4 | 161±20·7* | 135±17·1‡ |
| Leucine-2- | 0 | 19·2±2·4 | 27·5±2·3 | 33·4±4·5 | 39·5±5·6 | 40·6±6·2 | 43·8±3·5 |
| naphthylamidase | 0·3 | 18·2±2·1 | 28·7±2·0 | 31·6±3·8 | 41·9±4·1 | 48·5±5·4 | 54·0±2·7† |
| | 6 | 21·6±1·6 | 28·4±1·9 | 32·5±4·1 | 40·3±3·9 | 39·3±7·6 | 41·5±2·9 |
| DNA | 0 | | 6·2±0·75 | | | | 6·37±0·72 |
| μg/plate | 0·3 | | 6·4±0·80 | | | | 5·24±0·75 |
| | 6 | | 7·5±1·11 | | | | 4·29±1·24* |

The activities of each enzyme are expressed as nmol product formed by substrate hydrolysis/μg of DNA/hr. * $P<0·05$; † $P<0·01$; ‡ $P<0·001$.

**Table 4.** Lysosomal enzyme levels and DNA content of macrophage cultures exposed to Clofazimine and/or cycloheximide for 120 hr. Drugs were not added to control cultures which were treated in the same manner

| | Concentration of Clofazimine (μg/ml) | Cycloheximide (μg/ml) | |
|---|---|---|---|
| | | 0 | 0·5 |
| *N*–Acetyl β–D–glucosaminidase | 0 | 2176 ± 188 | 328 ± 24·3‡ |
| | 0·3 | 3425 ± 302† | 344 ± 36·9‡ |
| Cathepsin B | 0 | 63·7 ± 8·5 | 27·6 ± 3·7‡ |
| | 0·3 | 96·4 ± 12·7* | 29·2 ± 4·1‡ |
| β–glucuronidase | 0 | 65·3 ± 6·7 | 11·5 ± 1·3† |
| | 0·3 | 83·4 ± 4·2* | 11·7 ± 0·8‡ |
| DNA | 0 | 4·2 ± 0·4 | 4·0 ± 0·3 |
| μg/plate | 0·3 | 4·1 ± 0·2 | 3·9 ± 0·5 |

In the presence of Clofazimine a significant increase in lysosomal enzyme levels was observed; * $P < 0{\cdot}05$; † $P < 0{\cdot}01$. In the presence of Cycloheximide significant decreases in cellular levels of lysosomal enzymes as compared to cultures without Cycloheximide is demonstrated; ‡ $P < 0{\cdot}001$. The activities of each enzyme are expressed as nmol product formed by substrate hydrolysis/μg of DNA/hr.

*The effect of Clofazimine on the activity of lysosomal enzymes found in granular fractions*

The increased lysosomal enzyme level induced by Clofazimine on macrophage cultures can be explained in different ways. With the following experimental series we tried to evaluate two of the most relevant possibilities. A lysosomal enriched granular fraction was obtained from calf alveolar macrophages, as described previously. The fraction was preincubated with varying Clofazimine concentrations for 15 min and the activity of *N*–acetyl–β–D–glucosaminidase and β–galactosidase was assayed. No difference was found between control and Clofazimine treated fractions indicating that the drug, at the concentration employed, had no direct effect on the activity of the enzymes (Table 3). We could, therefore, not explain the enhanced lysosomal enzyme activity found in cultured macrophages by a direct effect of the drug on the enzymes studied.

*The effect of inhibition of protein synthesis on the increase of the lysosomal enzyme level induced by Clofazimine*

Macrophage cultures were exposed to 0·3 μg/ml of Clofazimine and/or 0·5 μg/ml of cycloheximide in order to determine if the induced increase of lysosomal enzyme level could be abolished by an inhibitor of the protein synthesis, Cycloheximide, at the concentration employed, has been shown to inhibit the protein synthesis of cultured macrophages, without any obvious cytotoxic effect (Einstein, Schneeberger & Coltens, 1976). The results obtained demonstrate that cycloheximide, in our experimental conditions, was capable of inhibiting the Clofazimine-induced increase in the level of lysosomal enzymes. The constant DNA content of culture plates as well as the morphological observations demonstrated no cytotoxic effect.

These results indicate that Clofazimine induces lysosomal enzyme synthesis at this concentration (Table 4).

## DISCUSSION

In the present study we have shown that Clofazimine induces an increase in the lysosomal enzyme level of cultured macrophages. A direct effect of the drug on the enzyme activities was not supported by our results. Clofazimine does not modify the enzyme activity of crude lysosomal fractions. Cycloheximide abolished the enhancing effect, suggesting that the drug induces an increased lysosomal enzyme synthesis. An alternative hypothesis, which could explain our results, may be that the drug induces a diminished rate of disposal of the lysosomal content of macrophages. Although

we have not evaluated this possibility experimentally in the present paper, preliminary results in our laboratory (unpublished) suggest that this is not the case. Our findings are supported by the results obtained by Conalty & Jackson (1962) studying the ultra-structural changes of macrophages following oral drug administration to mice. He and his colleagues observed an increase of the number, size and electrondensity of cytoplasmic inclusion bodies (phagosomes, lysosomes and phagolysosomes) as treatment progressed. At present we are studying the ultrastructure of Clofazimine-treated macrophage cultures in an attempt to correlate our biochemical data with the ultrastructural changes induced by the drug.

Although we found an increase in activity in macrophages from two different species and location (peritoneal macrophages in mice and alveolar macrophages in the calves) toxicity was present in high concentration only in the mouse macrophage population. The differences we observed could be due to (1) biochemical and functional differences between peritoneal and alveolar macrophages as previously reported (Oren *et al.*, 1963); (2) species differences; (3) peritoneal exudate cells were cultured in NBCS, which is known to induce pinocytosis and lysosomal enzyme synthesis in mice, while alveolar macrophages were cultured in homologous serum.

Our results could be relevant to the mechanism of Clofazimine in several ways. The drug concentration found to increase the lysosomal enzyme content of macrophages of two different species and sources is close to the therapeutic serum concentration reported by Banerjee *et al.* (1974) and Levy (1974). This suggests a general action of the drug on macrophages at the therapeutic concentration. Clofazimine-treated macrophages, through their increased lysosomal enzyme content, are probably more capable of killing intracellular parasites. Evidences in this direction have been reported by Cline (1970). The digestion of ingested particles, such as immune complexes will benefit from the action of Clofazimine on the macrophages lysosomal apparatus. The results presented in this paper were obtained with a simple experimental model macrophage culture. Studies with animals treated with Clofazimine searching for changes in the lysosomal enzyme level, necessary to support our hypothesis, are currently in progress in our laboratory.

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