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In Vivo Effect of Clofazimine in the Lysosomal Enzyme Level and Immune Complex Phagocytosis of Mouse Peritoneal Macrophages[1]

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In spite of the fact that clofazimine, 3-(*p*-chloroaniline)-10-*p*-chlorophenyl-2,10-dihydro-2-isopropylimine, was synthesized in the early 1960s ([2]) and that its antileprotic activity since then has been well documented ([3,13]), its mode of action remains largely unknown. We recently reported ([10]) that this drug, *in vitro*, at concentrations near the serum therapeutic levels increased the synthesis of lysosomal enzymes and the amount of labeled immune complexes (IC) phagocytosed ([11]) by nonstimulated mouse peritoneal macrophages. In this study, we report the action of the drug on macrophages obtained from mice fed by gavage with various drug concentrations. The results obtained further support our hypothesis that the beneficial effect of clofazimine in leprosy occurs through its action on the macrophage lysosomal apparatus.

MATERIALS AND METHODS

Experimental animals. Male Swiss mice weighing approximately 25 gm were obtained from the mouse colony established at the National Center for Scientific Research, Havana, Cuba.

Macrophage collection and culture. Groups of mice were fed by gavage during different time periods with various clofazimine concentrations. The clofazimine was a gift of Ciba Geigy, Ltd., Basel, Switzerland, and was dissolved in 0.3 ml of sunflower oil. At a given time, the mice were sacrificed by chloroform inhalation and the peritoneal cell exudate obtained as previously described ([7]). Briefly, 5 ml of M 199 (Gibco, U.K.) containing 20 U/ml of preservative-free heparin (Novo, Denmark), without serum, was injected into the peritoneal cavity. After gentle massage, the culture medium was aspirated and 5 ml aliquots of the collected fluid containing 0.5×10^6 cells/ml were distributed into 50 mm Petri dishes (Culture, grade, Nunc, Denmark). The plates were incubated 1 hr at 37°C in a humidified atmosphere of 5% carbon dioxide and air to allow the attachment of adherent cells. Nonadherent cells were removed by washing the plates four times with warm sterile saline. Morphologically more than 90% of the adherent cells were macrophages and 98% were viable by trypan blue exclusion. Fresh medium M 199 containing penicillin 100 U/ml and streptomycin 100 μg/ml supplemented with 10% v/v newborn calf serum (NBCS) was added to the plates. The plates were incubated overnight as indicated above. The next day the culture medium was aspirated, the cell layer washed twice with cold sterile saline, and 2.5 ml of saline containing 0.1% Triton X-100 was added to each plate. The cells were disrupted by scraping the plate with a silicone rubber policeman.

Enzyme assays. All enzyme substrates and chemicals were purchased from Sigma, London. The assays were performed under conditions giving linear release of product in relation to the amount of sample used and the time of incubation. β-Galactosidase was assayed by the method of Conchie, Findlay, and Levy ([6]). N-Acetyl-β-D-glucosaminidase was assayed by the method of Wollen, Heyworth, and Walker ([14]). Cathepsin C was assayed as suggested by Barrett ([1]). Substrates, pH, and buffers were used as previously reported by us ([10]).

Protein assay. The method of Lowry, *et al.* ([8]) was employed, using bovine serum albumin as a standard.

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TABLE 1. *Lysosomal enzyme[a] levels and protein content in peritoneal macrophages of mice treated with clofazimine for 21 days.*

Clofazimine concentration (mg/kg body weight/day)	N-Acetyl-β-D-glucosaminidase	β-Galactosidase	Cathepsin C	Protein (μg/plate)
0	1536 ± 138	98.2 ± 10.6	345 ± 42	690 ± 15
0.1	1498 ± 146	102.4 ± 11.5	336 ± 38	681 ± 66
1	2431 ± 202[b]	185.7 ± 16.6[b]	488 ± 57[b]	677 ± 98
10	2309 ± 219[b]	176.4 ± 17.3[b]	495 ± 51[b]	694 ± 73

[a] Enzyme activities are expressed as nmol of product formed by substrate hydrolysis/mg of protein/hr.
[b] Significantly increased compared to untreated controls; $p < 0.001$, Student's t test.

Purified IgG, antiserum and immune complexes. Rabbit IgG was obtained by the method of Chersi and Mage ([4]). Goat anti-rabbit IgG was obtained by repeated subcutaneous injections of 1 mg purified IgG in Freund's complete adjuvant. The animals were bled one week after the last injection. Antigen-antibody complexes were prepared at the equivalence zone. The precipitate was thoroughly washed with cold saline. The protein content of the immune complex (IC) was determined by the method of Lowry, *et al.* ([8]). Solutions were stored at 4°C for no longer than 3 days.

Iodination. Purified IgG was labeled with carrier-free ^{125}I (Radiochemical Centre, Amersham, U.K.) by the method of MacConahey and Dixon ([9]). Unbound ^{125}I was removed by means of gel filtration on Sephadex G-25, and IgG aggregates were eliminated by centrifugation at 15,000 *g* for 90 min. The specific activity of the IgG was 60 μCi/mg of protein. Each batch of radioactive IC had a specific activity of approximately 0.2 μCi/ml. Less than 2.5% of the radioactivity was soluble in cold 10% trichloroacetic acid (TCA). The labeled IgG was frozen in 1 ml aliquots until used. Each batch was used while the IC radioactivity was enough for the measurement of phagocytosis.

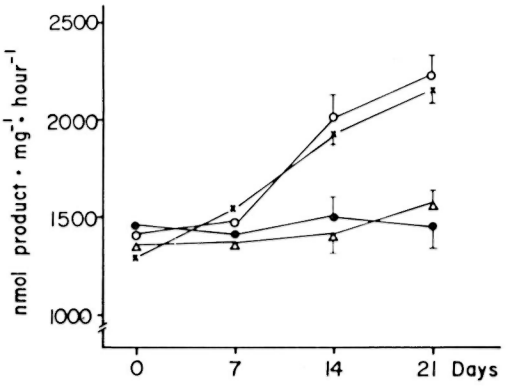
Phagocytosis assay. Culture medium was removed from the plates and fresh medium containing 20 μg/ml of ^{125}I-IC, supplemented with 10% NBCS, was added. Two hours later the medium was removed and the cell layer washed four times with warm saline. Two plates per clofazimine concentration were treated in the same manner except that they were kept at 4°C during the length of the experiment and washed with cold saline. In those plates, cell associated ^{125}I-IC was less than 2% of that phagocytosed in the experiment. Cells were removed from the plates as indicated above. Radioactivity was measured in a well-type scintillation counter.

Statistical tests. Quadruplicate cultures were done for each drug concentration in every experiment. Each experimental series described in this paper was repeated at least three times with the same results. Means and standard deviations were calculated after samples were shown to be homogeneous by calculations of coefficients of variance. The significance of differences was established by Student's t test.

RESULTS

We decided to test three clofazimine concentrations—0.1 mg, 1 mg, and 10 mg/kg body weight/day—since Shepard and Chang ([12]) reported that the two higher drug concentrations, when given to animals inoculated with *M. leprae* in the foot pad, were effective in preventing bacterial multiplication. Groups of mice were daily given the drug, dissolved in 0.3 ml of sunflower oil, by gavage. A control group received only the sunflower oil. At the beginning of the experiment and every week for three weeks, mice were killed by chloroform inhalation, the peritoneal cells obtained, and cultured as described above.

Drug effect on lysosomal enzyme level. Nonstimulated peritoneal macrophages obtained from mice that had received 1 mg and 10 mg clofazimine/kg body weight for 21 days showed a significant increase ($p < 0.001$) of the three lysosomal enzymes tested (Table 1). There was no difference in the protein concentration. Since N-acetyl-β-D-



THE FIGURE. Time-dependent effect of clofazimine treatment on cellular N-acetyl-β-D-glucosaminidase levels in macrophages of mice treated for different periods of time with the drug. Each value represents the means of four determinations. Enzyme activity is expressed as nmol of product formed by substrate hydrolysis/mg of protein/hr.

●—● = 0.1 mg/kg body weight/day; × — × = 1 mg/kg body weight/day; O—O = 10 mg/kg body weight/day; △—△ = controls.

glucosaminidase is an efficient lysosomal enzyme marker, time dependence of the increase was studied with this enzyme. The N-acetyl-β-D-glucosaminidase activity was studied weekly (The Figure). It was found that enzyme activity increased in macrophages obtained from animals that had received 1 mg and 10 mg clofazimine/kg body weight at 14 days, and at 21 days there was a further increase of the enzyme activity in both groups. Neither control groups nor those that had received 0.1 mg clofazimine/kg body weight showed an increase of the lysosomal enzyme activity.

Drug effect on immune complex phagocytosis. There was no difference in the amount of ^{125}I-IC phagocytosed by the control group and by those groups receiving various clofazimine concentrations during the first week. At the end of the second week, macrophages obtained from mice that had received 10 mg clofazimine/kg body weight phagocytosed more ($p < 0.001$) IC than those from other groups (Table 2). At the end of the experiment (three weeks), macrophages from mice that had received daily doses of 1 mg and 10 mg clofazimine/kg body weight showed a significant increase of ^{125}I-IC phagocytosed over the other groups.

DISCUSSION

When studying the mode of action of a drug sometimes experimental results obtained with simple *in vitro* models cannot be reproduced when animal models are utilized. In those cases, the *in vitro* results are frequently not relevant for comprehension of the drug's mechanism of action. Therefore, with the experimental series reported in this paper we aimed to evaluate the action of clofazimine on the macrophage lysosomal apparatus of mice that received the drug for up to three weeks. We assumed that if clofazimine modified the macrophage function in these animals, this time period would be sufficient for the expression of the drug's activity.

The results obtained by feeding mice clofazimine at concentrations of 1 mg and 10 mg/kg body weight/day demonstrated the same effects on macrophage function as those found *in vitro*. How to explain this? We have found no difference in the peritoneal cell adherence (data not shown) and in cell populations between control and clo-

TABLE 2. *Time-dependent effect of clofazimine on the phagocytosis of ^{125}I labeled immune complexes by peritoneal macrophages of mice treated with different levels of the drug.*

Clofazimine concentration (mg/kg body weight/day)	Cell radioactivity cpm/mg of protein (treatment time in days)			
	0	7	14	21
0	5148 ± 623	5135 ± 487	4780 ± 496	4878 ± 475
0.1	5332 ± 538	5276 ± 409	4978 ± 454	4675 ± 405
1	5298 ± 582	5289 ± 415	4897 ± 472	8398 ± 417[a]
10	5303 ± 611	5197 ± 493	8089 ± 631[a]	7781 ± 496[a]

[a] Significantly increased compared to untreated controls; $p < 0.001$, Student's t test.

fazimine-treated mice, although peritoneal macrophages obtained from clofazimine-treated mice were bigger and had more dense granules than macrophages obtained from control mice (data not shown). We have previously reported ([10]) that the drug does not have direct action on lysosomal enzyme activity and that the presence in the culture media of a protein synthesis inhibitor, cycloheximide, inhibits the increase of this activity. The above-mentioned experimental observations allow us to suggest that the increase of lysosomal enzyme activity is due to an increase of lysosomal enzyme synthesis induced by the drug. In an attempt to explain the increased phagocytic capacity found *in vitro* as well as *in vivo,* we studied macrophage Fc and complement receptors. Macrophages cultured in the presence of clofazimine or obtained from the peritoneal cavity of mice receiving the drug in the same manner as reported in this paper have more of these receptors than control macrophages (data not shown). This may explain the results reported here.

An increase in the time of exposure to a higher drug concentration does not result in a proportional increase of lysosomal enzyme level or phagocytic capacity. Results have been published demonstrating that clofazimine accumulates in the macrophage, and Conalty, *et al.* ([5]) have shown that prolonged exposure to clofazimine results in intracellular crystal deposition of the drug. Perhaps progressive accumulation of the drug may alter macrophage function in a manner different from that observed by us. The results obtained suggest that a continuous increase of the drug concentration over a longer time period does not necessarily lead to an enhanced macrophage function and could, in fact, be harmful to the cell.

These results add further experimental evidence to our suggestion that clofazimine's beneficial action on leprosy is at least partially mediated through an action of the drug on macrophage function.

SUMMARY

The effects of clofazimine on macrophages obtained from mice fed by gavage with various drug concentrations were studied. The results obtained demonstrated an increase in the activity of various lysosomal enzymes and in the amount of labeled immune complexes phagocytosed at drug concentrations of 1 mg/kg and 10 mg/kg body weight. This confirms and extends the effects reported by us of clofazimine's action on the lysosomal apparatus.

RESUMEN

Se estudiaron los efectos de la clofazimina sobre los macrófagos obtenidos de ratones tratados por vía oral con varias concentraciones de la droga. Los resultados obtenidos demostraron un incremento en la actividad de varias enzimas lisosomales y en la cantidad de complejos inmunes marcados fagocitados cuando la concentración de la droga se incrementó de 1 a 10 mg/kg de peso corporal. Esto confirma y extiende los efectos de la clofazimina sobre el aparato lisosomal que fueron publicados antes por nosotros.

RÉSUMÉ

On a étudié les effets de la clofazimine sur des macrophages obtenus chez des souris nourries par gavage avec différentes concentrations de ce médicament. Les résultats obtenus ont démontré une augmentation dans l'activité des diverses enzymes lysosomiques, de même que dans la quantité de complexes immuns marqués, et ceci pour des concentrations de médicaments allant de 1 mg/kg à 10 mg/kg de poids corporel. Ces observations confirment et élargissent la portée des effets rapportés antérieurement sur l'action de la clofazimine sur l'appareil lysosomique.

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