# Biochemical and Ultrastructural Study of the Relationship Between Lysosomal Enzyme Activities and Chemotherapy<sup>1</sup>

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One of the ultrastructural characteristics of lepra cells in human leprosy is the occurrence of opaque droplets around the bacilli (9, 12). Electron microscopic cytochemistry showed that these droplets contain acid phosphatase, and consequently they were named "lysosomal substances" (10). In addition it was observed that chemotherapy, especially with DDS, elicits the formation of the lysosomal substance, which may reflect an enhanced host-defense mechanism against bacilli (10).

On the other hand, bacillated histiocytes (murine lepra cells) in murine leprosy lesions usually lack opaque droplets (<sup>15</sup>). Furthermore, the cytotoxicity of murine leprosy bacilli is as low as that of human leprosy bacilli, differing from other bacterial infections. These lesions may, therefore, be useful to determine whether or not chemotherapy provokes the formation of opaque droplets in human lepra cells.

The present paper describes the morphologic and biochemical effects of some antileprosy drugs, such as 4,4'-diaminodiphenyl sulfone (DDS), Promin and streptomycin on lysosomal enzyme activities in murine leprosy lesions.

## MATERIALS AND METHODS

A total of 340 healthy white mice (strain MRT) weighing from 20 to 28 gm. were used in the following experiments.

Experiment 1. Ninety mice were inoculated in the peritoneal cavity with Mycobacterium lepraemurium (Hawaiian strain). Four months later 10 of these mice were killed and the liver, spleen, kidneys and mesenteric lepromas were removed. The remaining mice were divided into two equal groups. The mice of one group were injected intraperitoneally once a week with 0.2 ml. of Promin (400 mgm/ml.) for a three and a six week period. The other group was used for control as infected but untreated. At the end of each period the liver, spleen, kidneys and mesenteric lepromas were removed from 10 mice of each group.

Seventy healthy mice were used for normal controls, with the same procedures as noted above.

Experiment 2. One hundred and fifty mice were inoculated subcutaneously and intraperitoneally with *M. lepraemurium*.

After a lapse of two months the mice were divided into four equal groups, three receiving chemotherapy and the other none. The three chemotherapy groups were injected twice a week intraperitoneally, intramuscularly, and subcutaneously with 0.1 ml. of Promin, 0.1 ml. of DDS (50 mgm./ml.), and 0.1 ml. of streptomycin (50 mgm./ml.), respectively. At six and 19 weeks, subcutaneous lepromas, mesenteric lepromas and serum were removed from 10-15 mice. Thirty healthy mice were used as normal controls.

The isolated organs were immersed separately in beakers containing 0.25 M icecold sucrose. After the organs were weighed they were cut into small pieces with scissors and homogenized in a Potter-Elvehjem homogenizer with 10 volumes of 0.05 M acetate buffer, pH 5.0, containing 0.1 per cent Triton X-100, Following centrifugation at 12,000 x g for 20 minutes, the supernatant was used for enzymatic assays. The activity of acid phosphatase (EC 3.1.3.2),  $\beta$ -glucuronidase (EC 3.2.1.31), and cathepsin was determined by the methods of de Duve *et al.* (5). Since these hydrolases are mainly located in lysosomes

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(5, 13), the total activity of these enzymes in the supernatant was used to estimate the lysosomal enzymatic activity. Because of its high sensitivity, the activity of  $\beta$ -glucuronidase was the only one examined in sera.

Protein was determined by the method of Lowry *et al.* (<sup>11</sup>) using bovine serum albumin as a standard. Specific activity was expressed as  $\mu$ moles of the product per minute and per mgm. of protein.

Biopsy tissues from both the subcutaneous and the mesenteric nodules were fixed with buffered glutaraldehyde and examined by Ericsson and Trump's ultrastructural-cytochemical technic ( $^{6}$ ).

#### RESULTS

Biochemical assay. Table 1 shows hydrolase activity in different organs of healthy, infected, and Promin-treated animals (Experiment 1). Hydrolase activity in livers of infected mice was two to five times higher than in healthy mice, and one and a half to two times higher in the spleens of infected mice. Since the infected spleen weighed about two and one-half times more than the normal spleen, its total enzymatic activity was three to five times higher. The activity of  $\beta$ -glucuronidase and cathepsin in the kidneys of infected mice was one and a half to two and a half times higher; the acid phosphatase activity showed no significant increase.

The specific activities of the organs showed no appreciable effects after Promin injection. However, a slight increase of the enzymatic activity was evident in mesenteric lepromas treated with Promin.

Figures 1 and 2 show the effects of drug treatment on the hydrolase of subcutaneous and mesenteric lepromas (Experiment 2). During the course of infection specific enzyme activity increased in both control and treated (except for Promin-treated) animals and particularly in those with mesenteric lesions. The  $\beta$ -glucuronidase activity in sera of infected mice was two to six times higher than in that of healthy mice, indicating that the lysosomal enzymes from infected tissues were liberated into the sera.

Promin caused a peculiar effect on  $\beta$ -glucuronidase activity; e.g., while the

enzymatic level in both mesenteric and subcutaneous lesions decreased with time (6-19 weeks), the level in sera increased. It was also noted that the activity of the three enzymes in these lesions was lower at 19 weeks than the activity in untreated lesions. DDS increased the activity of all enzymes, especially in subcutaneous nodules after six weeks' treatment, whereas it was not remarkable in mesenteric lesions.

Streptomycin had a slight stimulating effect on lysosomal enzyme activity. Its effect was lower than that of DDS, especially at the beginning of the treatment.

The acid phosphatase activity in subcutaneous lesions of treated and untreated mice decreased during treatment, whereas that of mesenteric lesions increased significantly.

Electron microscopy. Murine lepromas in both subcutaneous and mesenteric tissues consisted of bacillated histiocytes, leukocytes, and mast cells (Fig. 3). Inside the membrane-limited vacuoles, a single or a group of bacilli were surrounded by an electron-transparent substance. Frequently moderately dense amorphous substances and fibrillar materials were observed in the vacuoles. In untreated animals opaque droplets were scarcely found in the cytoplasm of these bacillated histiocytes.

In DDS-treated animals, abundant opaque droplets occurred in the cytoplasm of histiocytes (Fig. 4). Fusion of opaque droplets around the bacilli was evident (Fig. 5); consequently, the large dense substances around the group of bacilli are interpreted as accumulations of small cytoplasmic opaque droplets. Acid-phosphatase activity was demonstrated in the opaque droplets around the bacilli (Fig. 5).

Promin injection elicited an increase of opaque droplets in murine lepra cells only at an early stage of treatment. However, the enhancement of opaque droplets by Promin is less than that caused by DDS treatment. The use of Promin injections for a prolonged period of time caused accumulation of bacilli, which were surrounded by a large amount of electron-transparent substances, as generally seen in human lepra cells.

Streptomycin-treated lepromas, com-

| lepromas in murine leprosy and the              |                                              |
|-------------------------------------------------|----------------------------------------------|
| 1, and mesenteric                               |                                              |
| spleen, kidnei                                  |                                              |
| indrolytic enzymes of liver,                    |                                              |
| TABLE 1. The specific activities of lysosomal h | ffect of Promin on the enzymatic activities. |

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|                                             |                   |      |       |      |      | DV7    | ndsond p   | atase X i            | - 0    |      |      |            |      |
|---------------------------------------------|-------------------|------|-------|------|------|--------|------------|----------------------|--------|------|------|------------|------|
| Enz                                         | yme tissue        |      | Liver |      |      | Spleen |            |                      | Kidney |      | Mese | nteric lep | roma |
| Conditions                                  | Week <sup>a</sup> | 0    | 3     | 9    | 0    | ŝ      | 9          | 0                    | 3      | 9    | 0    | 3          | 9    |
| 14                                          | - Promin          | 16.0 | 1.06  | 1.10 | 1.28 | 96.0   | 1.09       | 1.47                 | 16.1   | 1.65 | T    | 1          | 1    |
| Healtny                                     | + Promin          | Ĩ    | 66.0  | 1.15 | 1    | 0.91   | 1.19       | 1                    | 2.28   | 1.85 | 1    | 1          | 1    |
|                                             | – Promin          | 2.73 | 2.88  | 3.46 | 1.89 | 2.30   | 2.19       | 1.87                 | 2.02   | 1.66 | 1    | 1.49       | 1.48 |
| Intected                                    | + Promin          | Ĩ    | 3.18  | 3.17 |      | 2.27   | 2.22       | I                    | 1.95   | 2.44 | 1    | 2.38       | 2.34 |
|                                             |                   |      |       |      |      | B      | glucuronic | lase $\times 10$     | )-3    |      |      |            |      |
|                                             | - Promin          | 0.94 | 1.42  | 0.86 | 3.40 | 3.36   | 3.35       | 0.52                 | 0.72   | 0.75 |      |            |      |
| Healthy                                     | + Promin          | 1    | 1.26  | 96.0 | l    | 3.18   | 2.94       | 1                    | 0.85   | 0.64 | 1    | l          | 1    |
| Let a la l | - Promin          | 4.84 | 5.24  | 3.78 | 4.43 | 5.35   | 5.15       | 1.29                 | 1.55   | 1.83 | 1    | 5.80       | 5.09 |
| Intected                                    | + Promin          | 1    | 4.74  | 3.74 | 1    | 5.10   | 4.79       | 1                    | 1.96   | 1.82 | 1    | 5.61       | 7.20 |
|                                             |                   |      |       |      |      |        | Cathepsin  | 1 × 10 <sup>-2</sup> |        |      |      |            |      |
|                                             | - Promin          | 1.06 | 0.99  | 0.95 | 2.78 | 2.44   | 2.58       | 1.48                 | 2.22   | 1.69 | 1    | 1          | 1    |
| neariny                                     | + Promin          |      | 0.89  | 1.00 |      | 2.55   | 2.60       | Ŀ                    | 2.14   | 1.80 | t    | 1          | I    |
| 1.6.4.4                                     | - Promin          | 2.38 | 2.50  | 2.60 | 2.94 | 3.31   | 3.80       | 2.60                 | 3.43   | 3.25 | I    | 4.71       | 5.10 |
| Intected                                    | + Promin          | 1    | 2.53  | 2.58 | ]    | 3.52   | 3.95       | 1                    | 3.50   | 3.45 | 1    | 4.93       | 5.28 |

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weeks treated with Fromm. Specific activity is expressed as  $\mu$ moles of product (phosphate from  $\beta$ -glycerophosphate in acid phosphatase, phenolphthalein from phenolphthalein mono- $\beta$ -glucuronidase in  $\beta$ -glucuronidase



FIG. 1. Effects of streptomycin, DDS and Promin on the specific activities of hydrolases (acid phosphatase,  $\beta$ -glucuronidase, and cathepsin) of subcutaneous lepromas and sera in murine leprosy. (Serum  $\beta$ -glucuronidase activity is expressed as  $\mu$ moles of product per min. and per ml. of serum.)

posed of murine lepra cells, did not show any significant increase of cytoplasmic opaque droplets (Fig. 6). However, a small amount of dense substances with acid-phosphatase activity, was observed around the bacillary clump as well as in untreated lesions.

#### DISCUSSION

Elevated activities of the lysosomal hydrolytic enzymes in phagocytes have been demonstrated biochemically  $(^{3, 4, 13})$  and histochemically  $(^{2, 7, 8, 10})$  in tuberculosis and leprosy. On the basis of the bactericidal activity of cationic proteins contained in polymorphonuclear leucocyte lysosomes, it is suggested that these proteins work with hydrolytic enzymes to digest phagocytized bacteria  $(^{16})$ .

Our biochemical data indicate that lysosomal enzymes are undoubtedly enhanced in both bacillated and nonbacillated organs after murine leprosy infections, as observed in tuberculosis (<sup>3, 4, 14</sup>). The increased activity of  $\beta$ -glucuronidase in the sera of infected mice suggests that  $\beta$ -glucuronidase and possibly other lysosomal enzymes are released from the tissues into the blood stream during infection.

On the other hand, murine lepra cells usually lack typical lysosomal structures  $(^{14})$ , although the transitory appearance of the strongly acid-phosphatase-positive "peribacillary body" has been observed in the early stages of murine leprosy  $(^1)$ . It appears that in murine leprosy lysosomal hydrolases do not accumulate in lysosomes that can be visualized electron-microscopically as opaque droplets. Rather, major amounts of hydrolases may be dispersed throughout the cytoplasm and some may surround bacilli.

Streptomycin provokes increase of hydrolase activities, while no increase of opaque droplets is made evident in either lepra cells or nonbacillated histiocytes. It is possible that the enhancement of enzymat-



FIG. 2. Effects of streptomycin, DDS and Promin on the specific activities of hydrolases of mesenteric murine lepromas.

ic activities caused by streptomycin is represented only by a quantitative increase of free hydrolases in the cytoplasm.

On the other hand, DDS not only stimulates an increase of hydrolase activities in lesions, but also enhances formation of opaque droplets showing acid-phosphatase activity. It is interesting to note that Promin shows smaller effects on lysosomes, biochemically and morphologically, than DDS, although these two drugs are closely related chemical compounds. This fact implies that the enzymatic response of host cells may be unrelated to the chemical similarity of chemotherapeutic agents. Furthermore, DDS, which was the sole hydrophobic compound examined in the present study, may function physicochemically so as to accumulate enzymes in opaque droplets composed of lipoprotein (9).

It is to be noted that Palekar and Magar (<sup>13</sup>) recently reported that the specific activities of lysosomal enzymes from tissues of leprosy patients of all types decreased significantly after DDS treatment, showing

a tendency to attain normal values. Decrease of the enzymatic activity during the healing course of lepromas may be explained by the fact that histiocytes containing lysosomal enzymes disappear from lesions.

On the basis of biochemical and morphologic findings in the present study, we believe that antileprosy drugs, especially DDS and streptomycin, not only have direct bactericidal or bacteriostatic characteristics, but also elicit lysosomal hydrolase activities that indirectly may inhibit bacterial metabolism.

## SUMMARY

Activities of lysosomal enzymes (acidphosphatase,  $\beta$ -glucuronidase, and cathepsin) of liver, spleen, kidney, serum, subcutaneous lepromas, and mesenteric lepromas in murine leprosy were examined.

Tissues of infected mice had one and a half to six times higher specific activities than healthy mice.



Fig. 3. Murine lepta cells in mesenteric nodules formed four months after inoculation. A single bacillus or a group of bacilli are enclosed by vacuolar membranes (arrows). Fibrillar substances (F) can be seen in vacuoles. No opaque droplet is observed in the cytoplasm.



Fig. 1. Murine lepta cells in mesenteric nodules of four months-infected mice treated with DDS for one month. Note the occurrence of opaque droplets (OD) and dense substances surrounding bacillary clumps in the cytoplasm.



FIG. 5. Cytochemical demonstration of acid phosphatase activity in murine lepra cells under DDS treatment. Reaction products are abserved in both opaque droplets and in dense substances around the bacillus. Note the fusion of opaque droplets with the bacillus-containing vacuole (arrows). The specimen, removed by biopsy, is the same as that of Figure 4.

Effects of streptomycin, 4,4'-diaminodiphenyl sulfone, and Promin on both enzymatic activities and formation of opaque droplets in lepromas were observed. DDS treatment increased both enzymatic activities and opaque droplets in murine lepra cells, especially in subcutaneous lepromas. Streptomycin showed a slight stimulating effect on enzymatic activities without increasing the formation of opaque droplets. Promin induced an increase of enzymatic activities only in the early stages of treatment and did not provoke formation of opaque droplets.

Our data suggest that chemotherapy not only may play a bactericidal or bacteriostatic role, but also may enhance lysosomal enzymes that indirectly inhibit bacterial growth.

#### RESUMEN

Las actividades de enzymas lysosomales (ácido-fosfataso,  $\beta$ -glucuronidase, y cathepsin)

del hígado, baso, riñón, suero, lepromas subcutáneos, y lepromas mesentéricos en lepra murina fueron examinados.

Tejidos de ratones infectados tuvieron de uno y medio a seis veces mas altas actividades específicas que ratones sanos.

Efectos de la estreptomicina, 4,4'-diaminodiphenyl sulfona, y Promina en ambas actividades enzymáticas y en la formación de gotitas opacas en lepromas fueron observados. Tratamiento con DDS aumentó tanto las actividades enzymáticas como las gotitas opacas en células de lepra murina, especialmente en lepromas subcutáneas. La estreptomicina mostró un efecto estimulante ligero en actividades enzymáticas sin producir aumentos de formación de gotitas opacas. Promina produjo un aumento de la actividad enzymática solamente en las primeras etapas del tratamiento y no causó la formación de gotitas opacas.

Nuestra información indica que la quimoterapia no solamente puede desempeñar un papel bactericida o bacteriostático, sino también, liberar enzymas lysosomales que indirectamente inhiban el crecimiento bacteriano.



Fig. 6. Murine lepra cells in mescateric nodules of four months-infected mice under streptomycin treatment for one month. Dense substances (arrows) are found in vacuoles.

## RÉSUMÉ

On a examiné les activités des enzymes des lysosomes (phosphatase-acide,  $\beta$ -glycurconidase, et cathepsine) du foie, de la rate, du rein, du sérum, de lépromes sous-cutanés, et de lépromes mésentériques, dans la lèpre murine.

Les tissues de souris infectées présentaient des activités spécifiques plus élevées que ceux de souris saines, et ceci dans un odre de grandeur allant de un et demi à six fois.

On a étudié l'action de la streptomycine, de la 4,4'-diaminodiphenyl sulfone, et de la Promine, à la fois sur les activités enzymatiques et sur la formation de goutelettes opaques dans les lépromes. Le traitement par la DDS a entraîné à la fois une augmentation des activités enzymatiques et des goutelettes opaques dans les cellules lépreuses murines, particuliérement dans les lépromes souscutanés. La streptomycine a présenté un léger effet stimulant sur les activitiés enzymatiques, sans augmenter la formation des goutelettes opaques. La Promine n' a entraîné une augmentation des activités enzymatiques qu'au cours des stades précoces du traitement, et n'a pas provoqué la formation de goutelettes opaques.

Les données rapportées ici suggèrent que la chimiothérapie, non seulement peut jouer un rôle bactéricide ou bactériostatique, mais peut également stimuler les enzymes des lysosomes qui inhibent la croissance bactérienne de manière indirecte.

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