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STUDIES OF LYSOSOMAL ENZYMES IN MACROPHAGES

III. LYSOSOMAL ENZYME ACTIVITIES IN CULTURED MACROPHAGES INFECTED WITH SOME SPECIES OF MYCOBACTERIA*)

By

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ABSTRACT

The present study deals with changes of lysosomal enzyme activity in cultured mouse pertioneal macrophages infected with five species of mycobacieria. Infection with *M. tuberculosis* raised acid phosphatase activity in macrophages. *M. bovis* BCG and *M. microti* also caused rise in this enzyme activity but not β-glucuronidase activity. The cells infected with *M. leprae* showed decrease in both acid phosphatase and β-glucuronidase activities significantly. No change was observed in activity of acid phosphatase in the cells infected with *M. lepraemurium.* Infection with this microbe caused no change or decrease in both β-glucuronidase and N-Ac-β-glucosaminidase activities not only within the cells but also in the culture media. The pattern of enzymatic response was of similar quality between the in vivo and in vitro grown *M. lepraemurium.* Discussion was made about the difference in enzymatic response between the cells infected with tubercle bacilli and the cells with leprosy bacilli.

INTRODUCTION

It has been well known that some species of mycobacteria are refractory to the microbicidal and digestive attack of phagocytes and survive in the intracellular environment or even multiply[1-5]. As recently reviewed by Densen and Mandell[6], the resistant property of the acid-fast bacilli to phagocytes consists of three different mechanisms; the first, inhibition of degranulation (observed in *M. tuberculosis, M. bovis* and *M. microti*), the second, resisance to lysosomal attack (*M. leprae, M. lepraemurium* and *M. tuberculosis*), and the third, escaps from the phagosome (*M. bovis*). In spite of lots of works, the bactericidal mechanisms in macrophages have remained much less clear than those in polymorphonuclear leukocytes[7,8]. It is certain that lysosomal enzymes except lysozyme are not essential for killing microorganisms but essential for breaking down the integrity of the killed ones[9]. On the other

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Ⅲ．数種の抗酸菌感染培養マクロファージにおけるライソゾーム酵素活性

hand, previous reports demonstrated that so-called activated macrophages exhibiting antimicrobial activity contained large amounts of lysosomal enzymes[10], and that macrophages from *M. tuberculosis*-resistant rabbits possessed higher activity of acid phosphatase than those from sensitive ones[11]. These findings suggest the possibility of lysosomal enzymes as one of the candidates for the biochemical criteria in macrophages associated with the microbicidal action. The present communication deals with the biochemical changes brought about by interaction with intracellular parasites and phagocytes, with special reference to activity of some lysosomal enzymes in macrophages infected with mycobacteria in vitro.

MATERIALS AND METHODS

Microorganisms. Five species of mycobacteria were used in this study. *M. tuberculosis* (strain H37Rv) and *M. bovis* BCG (strain French) were maintained in our laboratory, and *M. microti* (TC-28, type strain) was supplied by Dr. Tsukamura (National Chubu Hospital, Japan). All these species were maintained with Proskauer and Beck medium (Difco Laboratories, U. S. A.) containing 1% special agar, Noble (Difco Laboratories, U. S. A.). *M. lepraemurium* (strain Kurume-42) was obtained from murine leproma developed by serial subcutaneous passages, and *M. leprae* from lepromatous tissues of leprosy patients without chemotherapy in Korea. *M. lepraemurium* (strain Hawaiian) grown in vitro was kindly supplied by Dr. Makino (Department of Leprology, Research Institute for Microbial Diseases, Osaka University, Japan).

Preparation of bacillary suspension. The bacilli grown in vitro were harvested after 2 to 3 weeks of growth and washed with phosphate buffered saline. Dispersed bacillary suspension was prepared by a teflon homogenizer. The bacillary suspension was centrifuged at 200 ×g for 5 to 10 min and the supernatant was suitably diluted with the culture medium. For the in vivo grown bacilli, the infected tissues were minced with scissors and mechanically disrupted by a glass homogenizer. The bacillary suspension was centrifuged and prepared in the same way as that of the in vitro grown one.

Macrophage culture. The method for macrophage culture was previously described in detail[12]. Briefly, peritoneal cells obtained from CF♯1 mice without simultant were allowed to settle on coverslips in multi-well plates (Linbro, Flow Laboratories Inc., U. S. A.) for 2 to 4 hr. The culture medium consisted of medium 199 (Nissui Seiyaku Co., Ltd., Japon), 20% fetal calf serum (Grand Island Biological Co., U. S. A.) and 100 U of penicillin per ml. Non-adherent cells were removed by washing with the medium and the adherent cells were further cultured overnight.

Infection with bacilli to cultured macrophages. The medium of cultured macrophages was replaced with the bacillary suspension. After macrophages were exposed to the suspension for 30 min (*M. tuberculosis*, *M. bovis* and *M. microti*) or 1 to 2 hr (*M. lepraemurium* and *M. leprae*), the cells were vigorously washed with the fresh culture medium several times to eliminate the bacilli remaining outside the cells. The average number of bacilli (Av. no. bacilli) phagocitized per cell at this time (0-time) was determined by examining at least 100 cells. The medium was changed every other day. At the end of the incubation period, the cells were washed with the medium three times and lysed by addition of 1 m*l* of 0.1% triton X-100 per well. Filtered with a membranous filter (pore size: 0.22 μm; Millipore Co., U. S. A.), the cell lysates and the medium were presented in biochemical assays but DNA was determined with samples prior to filtration. All the experiments were triplicated and the biochemical data are expressed as the mean and standard deviation.

Biochemical determinations. Lysosomal enzyme and DNA assays were previously described in detail[12]. Acid phosphatase, β-glucurocuronidase and N-Ac-β-glucosaminidase were assayed according to the method by Mead et al.[13], using 4-methylumbelliferone-conjugated substrates (Koch-Light Laboratories, Ltd., England). DNA was determined according to Hinegardner[14], using 2′-deoxyadenosine 5′-monophosphoric acid (d-AMP; Sigma Chemical Co., U. S. A.) as the standard. Enzyme activity was expressed as pmole per min per μg of d-AMP.

Enumeration of intracellular bacilli. Coverslips with infected macrophages were fixed

in absolute methanol and stained with Ziel-Neelsen method.

RESULTS

Infection with M. tuberculosis. The two

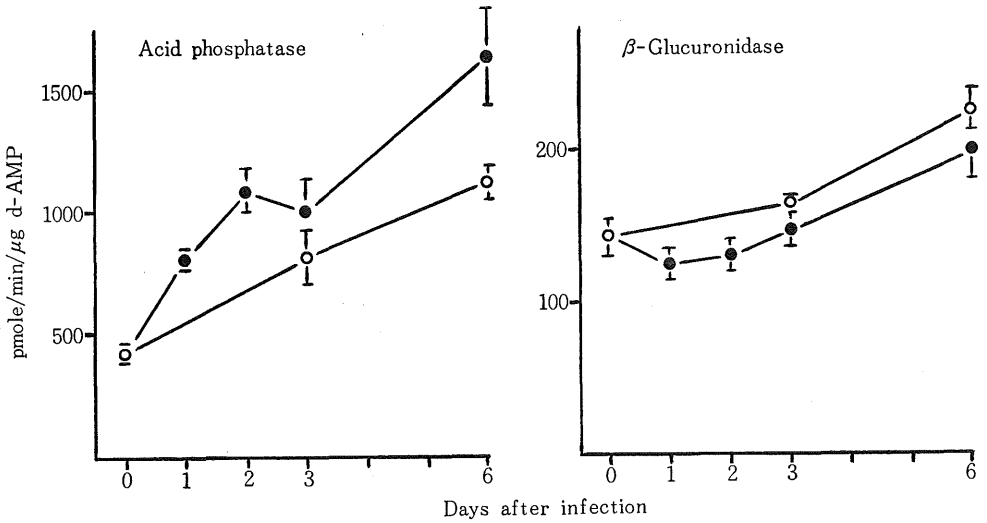

Fig. 1. Time course of lysosomal enzyme activities in macrophages after infection with *M. tuberculosis*. Av. no. bacilli phagocitized at 0-time was 1.0. Each activity was expressed as pmole per min per μg of d-AMP (mean±standard deviation). Closed circles represent enzyme activities in the infected macrophages, and open circles in the uninfected cells.

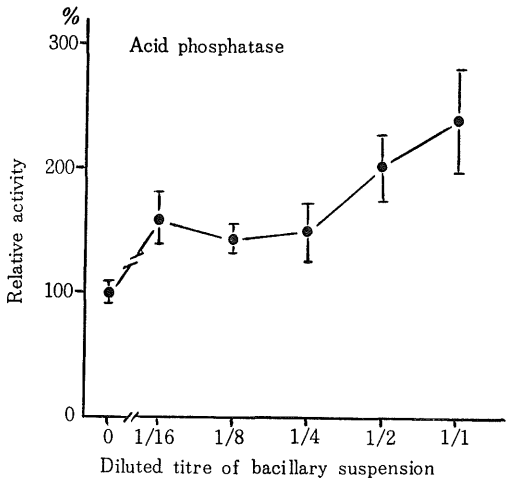

Fig. 2. Effect of the amount of infectious dose of *M. tuberculosis* on acid phosphatase activity in macrophages.
The bacillary suspension was adjusted to O. D. 0.02 at 540 nm and two-fold serial dilutions were made. Three days after infection with the bacilli, acid phosphatase activity in macrophages was measured. Each value was expressed as per cent of the enzyme activity in the uninfected cells (mean±standard deviation).

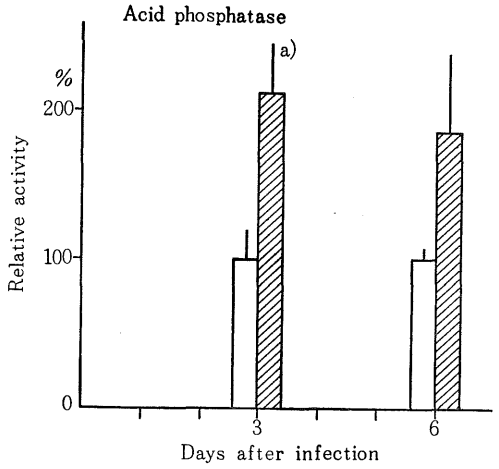

Fig. 3. Effect of *M. bovis* BCG infetion on acid phosphatase activity in macrophages.
Av. no. bacilli at 0-time was 0.9. Each value was expressed as per cent of the enzyme activity in the uninfected cells (mean±standard deviation). Hatched bars represent relative activities in the infected macrophages, and open bars in the uninfected cells.
a) $P<0.05$ compared with the uninfected cells.

lysosomal enzyme activities were traced 1, 2, 3 and 6 days after infection with *M. tuberculosis* to macrophages (Av. no. bacilli=1.0). As shown in Fig. 1, acid phosphatase activity in the infected cells on the lst day was already higher than that in normal cells and the increased ratio was steady until the 6th day, whereas β-glucuronidase activity was not.

The effect of the amount of infectious dose on acid phosphatase activity in macrophages was then observed. The bacillary suspension was adjusted to optical density of 0.02 at 540 nm and two-fold serial dilutions with the culture medium were made. Three days after infection with each dilution, the cells were harvested. The average number of bacilli per macrophage at 0-time was between 0.4 and 6.7. As shown in Fig. 2, the more heavily the cells were infected, the more highly the acid phosphatase activity increased.

Infection with M. bovis BCG or M. microti. Fig. 3 shows lysosomal enzyme activity in macrophages infected with *M. bovis* and Fig. 4 with *M. microti*. Infection with *M. bovis* BCG (Av. no. bacilli=0.9) caused a marked rise of acid phosphatase activity in the cells on the 3rd and the 6th day after infection with the bacilli. *M. microti*-infected macrophages (Av. no. bacilli=1.3) also showed higher acid phosphatase activity than control cells, but there was no significant difference in β-glucuronidase activity. The effect of *M. bovis* and *M. microti* on the lysosomal enzyme activity in macrophages was similar to that with *M. tuberculosis*.

Infection with in vivo grown M. lepraemurium or M. leprae. Macrophages were harvested on the 6th or 7th day after iefection with *M. lepraemurium* or *M. leprae*, and the acid phosphatase and β-glucuronidase activities were measured. The results are presented in Table 1. Neither acid phosphatase activity nor β-glucuronidase activity in the cells was influenced by the infection with *M. lepraemurium* *M. leprae*-infected macrophages showed no rise but fall in both enzyme activities, especially remarkable in β-glucuronidase. These results indicate that the response of the lysosomal enzymes in macrophages against *M. iepraemurium* or *M. leprae* is different from that

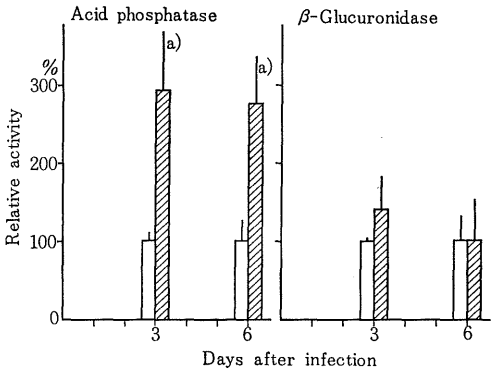

Fig. 4. Effect of *M. microti* infection on lysosomal enzyme activities in macrophages.
Av. no. bacilli at 0-time was 1.3. Each value was expressed as per cent of the enzyme activities in the uninfected cells (mean±standard deviation). Hatched bars represent relative activities in the infected macrophages, and open bars in the uninfected cells.
a) $P<0.05$ compared with the uninfected cells.

Table 1. Effect of in vivo grown leprosy bacilli infection on lysosomal enzyme activities in macrophages

	Groups	Av. no. bacilli at 0-time	Days after infection	Relative activities[a] of acid phosphatase	Relative activities[a] of β-glucuronidase
Exp. 1	Control	0	6	100±22	100±32
	M. lepraemurium	0.7	6	83±17(n. s.)	86±18(n. s.)
Exp. 2	Control	0	6	100±4	—
	M. lepraemurium	1.3	6	100±10(n. s.)	—
Exp. 3	Control	0	7	100±2	100±7
	M. leprae	4.2	7	87±4(<0.05)	47±2(<0.05)

a) Per cent of lysosomal enzyme activities in the uninfected macrophages.
Parentheses show significance compared with control; n.s.: not significant.

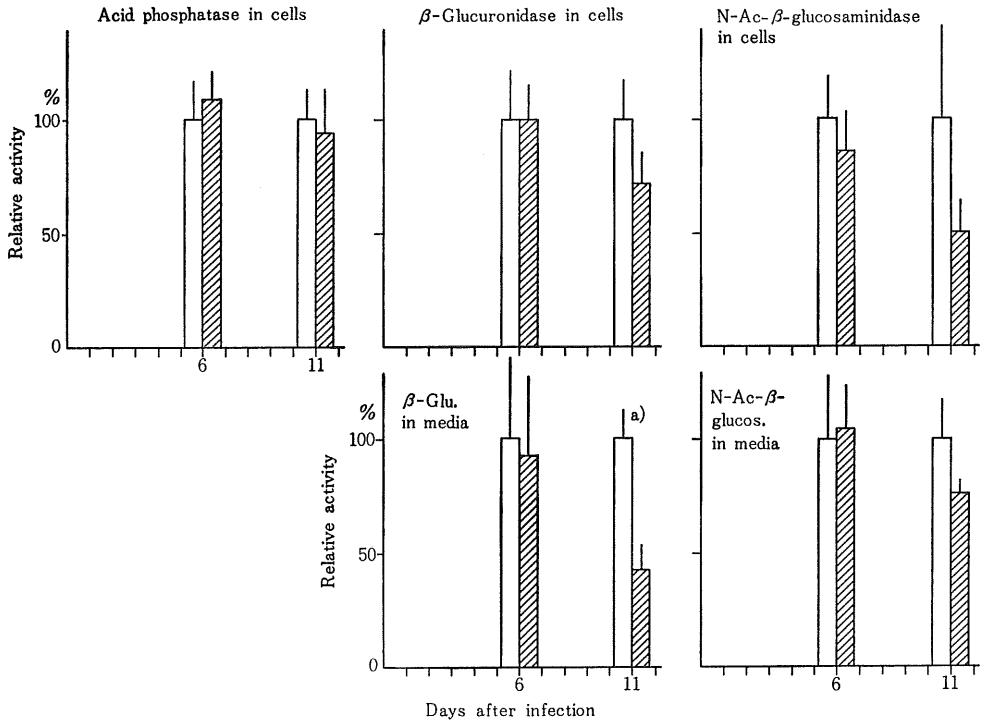

Fig. 5. Effect of in vitro grown *M. lepraemurium* on lysosomal enzymes in macrophages. Each value was expressed as per cent of the enzyme activities in the uninfected cells. Av. no. bacilli at 0-time was 11.4. Hatched bars represent relavive activities in the infected cells, and open bars in the uninfected cells.
a) $P<0.01$ compared with the uninfected cells.

of *M. tuberculosis*, *M. bovis* or *M. microti*.

Infection with in vitro grown M. lepraemurium. Fig. 5 shows the activities of three lysosomal enzymes in macrophages and those of two enzymes in the medium after infection with *M. lepraemurium* grown in vitro (Av. no. bacilli=11.4). Enzyme assays were carried out on the 6th and the 11th day after infection, because of the longer generation time of this microbe than other species of mycobacteria. No difference was observed in acid phosphatase activity between the infected and uninfected cells, whereas not significant but slight decrease in activities of β-glucuronidase and N-Ac-β-glucosaminidase was noted in the infected cells on the 11th day. Activities of β-glucuronidase and N-Ac-β-glucosaminidase in the medium were also measured. Since the medium had been changed 2 days before harvesting the cells, the extracellular activities presented in the figure show accumulated amounts secreted for 2 days from macrophages, unless the stability of enzymes in the culture medium is questioned. The pattern of enzymatic activity was well correlated with that found within the cells; that is, no change was seen in both the media on the 6th day but decrease was noticed on the 11th day in the medium from the infected cells.

DISCUSSION

Tissues obtained from tuberculous mouse lungs[15], BCG-induced pulmonary granuloma[16] and dermal BCG lesions[17] were reported to contain larger amounts of lysosomal enzymes. These authors suggest or speculate that the increased enzyme activity might be attributable to that of macrophages participated in the lesions. Althogh many works on lysosomal enzyme activity in macrophages or monocytes from mycobacteria-treated animals have been carried out[15,16,18-24], the results were divergent. There were differences in animal species, virulence and administration root of bacilli, interval

of infection and origin of macrophages. Some reports demonstrated that several lysosomal enzyme activities in alveolar macrophages from rabbits were raised by intravenous injection with heat-killed BCG but not by subcutaneous injection and intratracheal challenge with heat-killed BCG[16], or intradermal vaccination with live BCG even accompanied with revaccination[23]. These findings suggest that the differences in the enzymatic response conclusively reach the variety of local or systemic responses against the bacilli related to the intensity of cellular immunity, and further reach different modes of participation of macrophage in the responsive sites. The report by Pantalone and Page[25] that lymphokines stimulated the production and secretion of lysosomal enzymes by macrophages but reduced the enzyme activities within the cells is of interest. It has generally been recognized that intravenous vaccination with BCG increases lysosomal enzyme activity in both alveolar and peritoneal macrophages[16,22-24]. However, in these in vivo experiments, macrophages are allowed to be influenced not only by the bacilli themselves but also by factors derived from host's response against the bacilli. Therefore, the present study was carried out to see any change in intracellular activity of lysosomal enzymes resulting from the interaction with cultured macrophages and mycobacteria in vitro. Infections with *M. tuberculosis*, *M. bovis* BCG and *M. microti* raised acid phosphatase activity in macrophages but not β-glucuronidase activity. Suter and Hulliger[26] reported that BCG-infection of cultured rabbit macrophages resulted in an increase of acid phosphatase activity after 24hr, and Mizunoe and Dannenberg[23] observed no change in esterase and proteinase activities after 48 hr with BCG-infection. The present data support these results, and reveal that increased acid phosphatase activity in the BCG-infected macrophages sustained under longer cultivation. On the other hand, infection with in vivo grown *M. lepraemurium* caused no change in acid phosphatase and β-glucuronidase activities, but that with *M. leprae* reduced both the two enzyme activities. There are two noteworthy points in the interpretation of this matter. The first is concerned with the cellular viability. The number of adherent cells in the infected group was as many as or slightly more than that of the uninfected control group according to DNA assay (data is not listed). In addition, macrophages infected with the bacilli were observed microscopically to be more increased in cell size and ruffled membrane activity than the uninfected cells. These findings seem sufficient enough to deny the possibility that infection with the bacilli lowered the cellular viability. Another point is an error induced by contamination of cellular components from hosts since the in vivo grown bacillary suspenision were prepared from lepromatous tissues. Subsequent investigation with in vitro grown *M. lepraemurium* demonstrated no participation with cellular components of host origin; the pattern of enzyme activity in the cells infected with in vitro grown *M. lepraemurium* was the same as that with in vivo grown organisms at the same incubation period and further incubation reduced the activities of β-glucuronidase and N-Ac-β-glucosaminidase. The result indicates that *M. lepraemurium* and *M. leprae* produce the similar enzymatic response in macrophages. Further, decrease in activities of β-glucuronidase and N-Ac-β-glucosaminidase was not only observed in the cells but also in the culture media.

It is known that there is difference of virulence or infectivity between the in vitro and in vivo grown bacilli. Kanai[27] observed that the in vivo grown tubercle bacilli were coated with acid phosphatase derived from the host. However, no difference in the enzymatic response was found between the sources of *M. lepraemurium* in the present experiment. Kanai[28] also claimed that pH value of acid phosphatase specific for tubercle bacilli was between 5.6 and 6.5 with a single steep peak and was negligible below 5.0. In the present study, acid phosphatase activity was assayed at pH 5.0, and the infection of tubercle bacilli increased the enzyme activity in the cells even with very mild infection. Therefore, the enzymatic response by infection with tubercle bacilli is considered to be independent of the bacilli-specific enzyme activity.

It has been observed that activity of lysosomal enzymes, particularly acid phosphatase, in cultured mouse peritoneal macrophages is increased by endocytic stimuli which are completely degradated by the cells[29,30]. In contrast, non-digestable particles have been considered to be

ineffective as potentiators of lysosomal enzyme activity. Recently, Mørland and Mørland[30] demonstrated that latex or carbon particles caused no change in acid phosphatase activity but increase of cathepsin D and β-glucuronidase activities. Intracellular parasites are primarily nondigestable particles. Tubercle bacilli survive and multiply vividly in phagocytes standing against digestion by the cells. However, some metabolites secreted from the bacilli may become ingestable and result in increasing the activity of intracellular enzymes such as acid phosphatase. In addition, *M. tuberculosis* is well known to produce cytotoxic cord factor. On the contrary, leprosy bacilli are nontoxic to the host cells and produce much less metabolites with slower multiplication than the other mycobacteria. The killed bacilli in the tissues of patients with lepromatous leprosy under effective chemotherapy are not easily eliminated for a long period. These evidences indicate that leprosy bacilli are strongly nondigestable, which might pose one possibility to explain the difference in the enzymatic response between leprosy bacilli- and tubercle bacilli-infected cells.

Some observations are relevant in considering the meanings of enzymatic response in the cells infected with leprosy bacilli. According to Dwyer and his associates[31-33], *Leishmania donovani* survives and multiplies in phagolysosomes, and characteristically its surface is coated with glycoprotein. They speculated that such a surface layer protected this organism from degradation by lysosomal enzymes. Avila and Convit[34] reported that glycosaminoglycan inhibibed lysosomal enzyme activities at low pH. Leprosy bacilli also can live in phagolysosomes[35-37], and the surface is surrounded by an electrontransparent zone in the infected cells[35-39]. Possible similarlity of the biological significance of this material around the leprosy bacilli to that of *L. donovani* is presumed. Brown et al.[36,40] proposed an opinion that lysosomal enzymes might provide nutrients beneficial for the growth of *M. lepraemurium* because of the excellent multiplication in lysosome rich cultured fibroblasts.

Although a similar electron-transparent zone has been observed on *M. tuberculosis*[5,41] and *M. microti*[37] in the infected cells, these bacilli are different in the intracellular localization from leprosy bacilli and reside within phagosomes without lysosomal fusion[37,41]. Armstrong and D'Arcy Hart[42] observed that *M. tuberculosis* survived in phagosomes even when lysosomal fusion occurred. No. definitive interpretation is available so far as to why these mycobacteria are capable of intracellular survival. The present study may provide a clue for explaining the varieties of intracellular resistance of mycobacteria.

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