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Suppressive Activity of Streptomycin on the Growth of *Mycobacterium lepraemurium* in Macrophage Cultures[1]

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The effect of streptomycin on the growth of an obligate intracellular bacterium was studied in a new host-parasite cell system. The system consisted of *Mycobacterium lepraemurium* grown in cultures of mouse peritoneal macrophages. Since these organisms do not grow in bacteriological media, the influence of extracellular bacterial growth can be ruled out. The suppressive activity of streptomycin was observed in a total of five experiments. At the end of 4 weeks, the average number of organisms per macrophage for the controls was 65.7; for cultures with streptomycin at concentrations of 0.5, 1, 5, 10, 50, and 100 μg/ml of medium, it was 45.4, 38.3, 28.7, 22.7, 13.4, and 8.2, respectively. A good dose-response relationship was evident. *M. lepraemurium* which had been treated in macrophage cultures with various concentrations of the antibiotic for 6 to 8 weeks was used to infect fresh macrophages. These cultures were in turn treated with streptomycin. Resistance of the organisms to streptomycin did not occur.

Streptomycin was known to exhibit no antimicrobial activity on the intracellular growth of various species of bacteria kept in cell cultures (12, 20). It was postulated that this antibiotic is incapable of penetrating the host cells and, for this reason, streptomycin was used to selectively check the extracellular growth of facultative intracellular bacteria in studies of host-parasite relationships (2, 6, 8-10, 13, 19). However, this hypothesis was not uniformly supported by subsequent investigations. Reports of varying activity of streptomycin on intracellular parasites appeared in the literature. Many of these deal chiefly with uninhibited extracellular bacterial growth which is eventually phagocytized by host cells, simulating a true intracellular growth (3).

Recently a new cell system was developed in this laboratory, which employed an obligate intracellular bacillus, *Mycobacterium lepraemurium*, in cultures of mouse peritoneal macrophages (5). Since these organisms do not grow in bacteriological media, the influence of extracellular bacterial growth can be ruled out in this system.

My report presents results of studies on the suppressive activity of streptomycin upon *M. lepraemurium* in macrophage cultures.

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MATERIALS AND METHODS

Techniques for the cultivation of *M. lepraemurium* in cultures of mouse peritoneal macrophages were reported previously (4, 5). Briefly, peritoneal exudate was harvested from female ex-breeders of the general purpose strain of National Institutes of Health Swiss white mice. Macrophages were maintained as Leighton tube monolayers in a medium consisting of 40% horse serum, 50% NCTC 109, and 10% of a 1:5 dilution of beef embryo extract and liver extract (L-fraction), 1 mg/ml. Medium was renewed twice a week. Cultures were kept at 37 C in an atmosphere of a 5% CO_2-air mixture.

The Hawaiian strain of *M. lepraemurium*, which has been maintained in serially transferred macrophage cultures in this laboratory since 1964, was used in this study. Organisms were harvested from 5- to 6-week-old cultures. Macrophages were scraped off with a rubber policeman and homogenized in NCTC 109 medium in a Ten Broeck tissue grinder in order to release the bacilli. Infection was made by introducing a suitable number of organisms into the culture at 1 day after establishment of the monolayer. Phagocytosis of organisms was completed in 1 day. Streptomycin sulfate (E. R. Squibb & Sons, New York) was added 24 hr after infection in order to prevent direct contact of the antibiotic with nonphagocytized organisms. To preserve the potency of streptomycin, small portions of solution were kept frozen and 1 portion was used for

TABLE 1. *Effect of streptomycin on the growth of M. lepraemurium in macrophage cultures*[a]

Incubation time[b]	Control	Concn of streptomycin (μg/ml)					
		0.5	1	5	10	50	100
1 day	4.4	4.2	4.0	4.0	4.6	4.0	4.2
1 week	6.8	6.2	7.6	5.1	7.0	6.3	6.7
2 weeks	15.8	11.6	11.6	10.3	9.5	8.2	6.2
3 weeks	34.3	17.0	14.3	15.8	11.0	10.8	7.6
4 weeks	65.7	45.4 (30.9)	38.3 (41.7)	28.7 (56.3)	22.7 (65.5)	13.4 (79.6)	8.2 (87.5)
5 weeks	57.8			21.6 (62.6)			

[a] Values are expressed as number of organisms per macrophage. Percentage of growth reduction is expressed parenthetically.

[b] Five experiments were performed for each, except for 5 weeks, for which six were performed.

each renewal of medium. Penicillin was not added in these experiments.

Leighton tubes containing cover slips (8 by 22 mm) were used. Fixed, stained cover slips were examined at 1 day after infection and at 1-week intervals thereafter in a period of 4 to 5 weeks. The method for enumeration of organisms has been reported previously (5).

RESULTS

A total of five experiments were performed. Cultures in the first three experiments were infected with an average of two to four organisms per macrophage with a phagocytic rate (cells infected with the organisms) of 40 to 60% of the cell population. Macrophages in the other two experiments were infected with a larger number of organisms (five to eight per macrophage) and maintained a phagocytic rate of 80 to 90%. Six concentrations of streptomycin were used, 0.5, 1, 5, 10, 50, and 100 μg per ml of medium. Enumeration of organisms was made at 1 day after infection and at 1-week intervals thereafter for 4 to 5 weeks (Table 1).

In the control cultures to which no drugs were added throughout the experiments, the number of organisms increased more or less steadily. Growth of organisms was slightly faster in the heavier than in the lighter infection. The average number of organisms increased from 4.4 to 65.7 at the end of 4 weeks. Suppressive activity of streptomycin was observed in all five experiments. All six concentrations of streptomycin showed suppression, the degree of which appeared proportional to the concentration. In comparison to the growth of controls, a reduction of 30.9% in bacillary population was observed in cultures treated with the smallest concentration of streptomycin, and one of 87.5% in those treated with the greatest concentration. A good dose-response relationship was evident (Fig. 1).

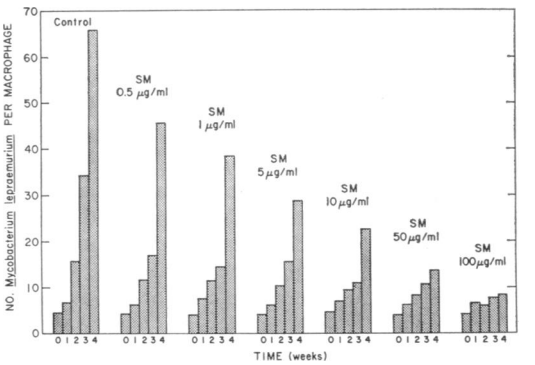

FIG. 1. *Dose-response relationship of streptomycin on the growth of M. lepraemurium in macrophage cultures. Bars represent average data of five experiments.*

Streptomycin activity was assessed after a 5-week cultivation in experiments 2 and 3 above. For comparison, the 5-week data from additional experiments 6, 7, 8, and 9 were included. Experiments 6 and 7 had been infected with an average of 3.1 organisms per macrophage and experiments 8 and 9 with an average of 6.1 and 7.8 organisms per macrophage, respectively. Only one concentration of the antibiotic, 5 μg/ml, was used. At 5 weeks, a reduction of 62.6% of the bacillary growth was observed, in comparison to a 56.3% reduction observed at the end of 4 weeks (Table 1).

Note that all macrophages in these experiments were maintained in good condition throughout the period of observation. Streptomycin did not reveal any toxic effect on host cells even with the highest concentration of the drug. This seems to rule out the possibility that high concentrations of streptomycin might cause host-cell damage which could facilitate penetration.

The pattern of bacteriostatic activity of strep-

TABLE 2. *Absence of resistance of M. lepraemurium to streptomycin in macrophage cultures*

Original culture	Transfer[b]	Concn of SM in final culture	No. of organisms per macrophage	
			At 1 day	At termination
SM,[a] 5 μg/ml, for 40 days	SM, 5 μg/ml, for 35 days	No SM	3.9	114.4
		SM, 5 μg/ml	3.1	21.8
SM, 10 μg/ml for 47 days	No SM for 45 days	No SM	8.4	45.0
		SM, 5 μg/ml	10.0	31.9
		SM, 10 μg/ml	9.9	35.1
		SM, 50 μg/ml	8.5	24.6
SM, 50 μg/ml for 56 days	No SM for 43 days	No SM	8.2	43.5
		SM, 10 μg/ml	7.1	14.0
		SM, 50 μg/ml	8.7	10.5
		SM, 100 μg/ml	7.5	12.5

[a] SM, streptomycin.

[b] For original culture concentrations of 10 and 50 μg/ml, bacilli were transferred a second time and cultured for 21 days at the three concentrations of streptomycin.

tomycin exhibited in the macrophage—*M. lepraemurium* cell system differed from that shown by other acid-fact organisms grown in bacteriological medium. In the former, a wide range of concentration, 0.5 to 100 μg/ml, was adequate for partial to complete inhibition, whereas in the latter a very small concentration, 0.6 μg/ml, was adequate for complete suppression (16)). One factor which might be responsible for the differences is possible emergence of resistance to streptomycin by intracellular *M. lepraemurium*. It was known that development of resistance to streptomycin is a rapid process in acid-fast organisms. Showacre et. al. (18) observed the emergence of resistance in *Salmonella typhosa* in L-cell cultures under phase contrast microscopy. Three experiments were performed to determine if resistance had emerged in the intracellular *M. lepraemurium* (Table 2).

In the first experiment, infected cultures were treated with 5 μg of streptomycin per ml for 40 days. Fresh macrophages were then infected with organisms from these initial cultures (the first transfer). These new cultures were also treated with 5 μg of streptomycin per ml. Marked suppression of bacillary growth, similar to the activity shown in the above experiments (Table 1, 5 weeks), was observed. This indicated no emergence of resistance to streptomycin after 40 days.

A higher concentration of streptomycin, 10 μg/ml, was used in the second experiment. There were only a few organisms after 47 days in the original culture. The organisms were then transferred to fresh macrophages with the omission of streptomycin (first transfer). A second transfer was made 45 days later, and these cultures were treated with three different concentrations of streptomycin for 21 days. Although definite suppression was observed with all three concentrations, streptomycin appeared to lack the potency shown at the end of 3 weeks in Table 1.

However, this was not true in the third experiment in which the cultures were treated with a still higher concentration of streptomycin, 50 μg/ml. After 56 days in the original culture with streptomycin and 43 days in the first transfer without streptomycin, the organisms were finally treated in the second transfer with the three concentrations of the antibiotic for a period of 21 days. Marked suppression of bacillary growth, which appeared to approach the activity shown in Table 1 at the end of 3 weeks, was observed with all three concentrations. It is believed that a persistent and irreversible resistance of the intracellular *M. lepraemurium* to streptomycin did not emerge in the present study.

DISCUSSION

The lack of development of resistance of *M. lepraemurium* against streptomycin is rather surprising. In the study of growth of *S. typhosa* in L-cell cultures, Showacre et. al. (18) observed extensive over-growth of streptomycin-resistant bacilli within 1 to 6 days. The resistant organisms are present in the seed inocula and emerge early in the course of the experiments. In my study, some degree of resistance of *M. lepraemurium* seemed to develop with a concentration of 10 μg of streptomycin per ml, but not with larger or smaller concentrations. The inconsistency in development of resistance may be related to the growth rate of the organisms. *S. typhosa* are rapidly growing organisms. A larger number of resistant bacilli would soon accumulate in the culture and be easy to detect. On the other hand, the growth of *M. lepraemurium* is very slow with a generation time of approximately 7 days in macrophages (5). The number of resistant *M.*

lepraemurium accumulated would be too small to be readily detectable in the present cell system. Alternatively, a reversal of resistance might also occur during the long-term process for detection of resistance. I observed no emergence of a persistent or irreversible resistance of *M. lepraemurium* to streptomycin.

Other factors that might have caused the wide range of antibiotic activity may be mentioned as follows: (i) lesser concentration of antibiotic within host cells than in the medium; (ii) partial inactivation of the antibiotic within host cells; and (iii) organisms damaged by streptomycin which, although incapable of growing in bacteriological media, might grow within host cells where better nutrients for growth could be obtained. Clearly more information is needed for further understanding of the growth pattern of this slow-growing obligate intracellular organism in tissue culture.

My experiments demonstrated the suppression of *M. lepraemurium* by streptomycin and indicated that the antibiotic enters the cell to accomplish this effect. An alternate possibility would be that the bacilli were egested, attacked extracellularly by streptomycin, and then reingested by the host cells. If egestion of organisms is a constant feature, the egested organisms should be taken up by uninfected macrophages. This would be reflected in an increase in infected cells. In my study, the rate of phagocytosis remained fairly constant throughout the experiments. Unlike tubercle bacilli which caused lysis of host cells thereby liberating the bacilli, *M. lepraemurium* did not have a destructive effect upon host cells. Uninfected macrophages were constantly observed in the vicinity of infected cells that had filled with numerous bacilli. In the macrophage system, *M. lepraemurium* grew only intracellularly. Streptomycin was added when the process of phagocytosis was complete, leaving little chance for organisms to be exposed to the antibiotic. Organisms were maintained within host cells throughout the experiments with little evidence of engagement in any perpetual ingestion-egestion phenomenon.

The effect of streptomycin on intracellular parasites in tissue cultures has been studied in many laboratories during the past two decades. Various types of host cells and species of organisms were employed. Because results differed so greatly among investigators, it is difficult to make a generalization regarding the intracellular action of streptomycin. At one extreme, suppressive activity of the antibiotic was found in a concentration as small as 0.5 μg/ml (J. C, Nagel, Jr., Bacteriol. Proc., p. 93, 1962), while at the other extreme, a concentration as great as 2,000 μg/ml (16) was found inactive. Although detailed discussion of the history of the subject is lengthy, the major problems involved in these studies may be summarized as follows.

Macrophages have been used extensively for host-parasite studies. Unfortunately, these cells could not be maintained in good condition in the early days. Many cells were lost during the first few days of cultivation (the maturation period), and deterioration of remaining cells became evident shortly thereafter. This was particularly true in experiments of Suter (20) in which the macrophages were not only in poor condition but were actually replaced by a type of sheet-forming, mesothelial-like cells after 4 to 7 days of cultivation. Moreover, the acid-fast staining used in his preparation for tubercle bacilli was inadequate to the degree that nearly 50% of the organisms appeared in the black and white photomicrograph as unstained white rods. This was even more evident in his colored slides (personal communication). One wonders whether the red or the colorless rods were used for evaluation of drug activity. Yet Suter's findings have led many investigators to use streptomycin for elimination of extracellular growth in host-parasite studies.

The cell-line cultures, such as HeLa, L, J-111, Henle epithelial, rat fibroblasts, human amnion, Chang's liver cells, etc., were used for studies of infections with various species of bacteria. The main disadvantage of these cultures was the difficulty in attaining phagocytosis of organisms. Certain batches of dog or horse serum (17) enhanced phagocytosis to a certain extent, leaving the remainder of organisms to multiply in the extracellular environment of the culture. Multiplication and subsequent ingestion of extracellular organisms may have resulted in an increase in the number of bacilli within the cells, simulating a true intracellular multiplication.

Methods for eliminating extracellular organisms have been developed such as washing infected cells by frequent renewal of medium (7) or by continuous flow of fresh medium into the culture (1). However, washing was considered ineffective by Machaness (11) and by Brumfitt et al. (3) for removing extracellular organisms, especially those immobilized on a glass surface far from the reach of phagocytic cells or those adhering to the surface of macrophages. Antiserum was also used to eliminate extracellular organisms. The results were conflicting. Inhibition of growth was observed in both intracellular and extracellular organisms by one group of investigators (15), in only extracellular organisms by a second (S. C. Nagle, Jr., Bacteriol. Proc. p. 93, 1962), and in no manner by a third (14).

Observation periods were too brief to simulate an in vivo infection.

Lack of studies on the dose-response relationship of the antibiotic caused conflicting interpretations of data.

These difficulties might have been resolved by using the macrophage-*M. lepraemurium* cell model used in the present study. The macrophages were maintained in good condition from the beginning of cultivation without passing through the period of maturation. Phagocytosis of organisms was very thorough. All inoculated organisms were found inside the macrophages. It was possible to maintain the macrophages in good condition for several months, which was much longer than the time required for the study. The period of observation was 4 to 5 weeks, which is sufficient to simulate an actual infection in vivo. There was no growth of extracellular organisms since *M. lepraemurium* does not grow in any known bacteriological medium. Moreover, a good dose-response relationship was observed in repeated experiments in the present study. With this cell model, a concentration of streptomycin as small as 0.5 μg/ml in the medium showed suppressive activity.

Finally, the wide range of drug-response I found might have played an important role in causing the differences in results observed among various investigators. Some investigators might have observed the partial suppressive activity of the small concentration of streptomycin. Others might have noticed only the more marked suppression from the larger concentrations of the antibiotic. This illustrates the importance of dose-response relationship studies.

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