Growth of *Mycobacterium lepraemurium* in Diffusion Chambers Containing Human Embryonic Skin Cells and in Cell-free Chambers

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SUMMARY

Mycobacterium lepraemurium grew in diffusion chambers containing human embryonic skin cells maintained in mice, guinea pigs and Petri plate monolayer cultures of human embryonic skin cells. Growth also occurred in diffusion chambers without skin cells maintained in mice and guinea pigs. A 28·8-fold increase in the number of bacilli was obtained when chambers containing skin cells were incubated in mice for 50 days and a 14·2-fold increase when incubated in guinea pigs. Significantly, chambers without skin cells gave a 12·2-fold increase in the number of acid-fast bacilli when maintained in mice for 50 days but only a 5·9-fold increase when maintained in guinea pigs for 50 days.

INTRODUCTION

The reproducible cultivation of *Mycobacterium lepraemurium* *in vitro* has been achieved only in tissue cultures of rat or mouse origin (Hawking, 1958; Wallace, Elek & Hanks, 1958; Rees & Garbutt, 1962; Chang, Andersen & Vaituzis, 1967; Matsuo, 1970). Recently, a new technique for growth studies with *M. lepraemurium* was developed in our laboratory (Rightsel & Wiygul, 1971). Multiplication of the organisms was demonstrated in diffusion chambers containing LM mouse fibroblasts or mouse peritoneal macrophages maintained in mice and guinea pigs and *in vitro* in Petri plate monolayer cultures of the same cell type inoculated into the chambers. Evidence also was presented for the growth of *M. lepraemurium* in cell-free chambers maintained in mice and guinea pigs. This was the first report on the growth of this organism in diffusion chamber systems or in a cell-free environment.

The primary objective of this present study was to determine if cells derived from a species other than the natural hosts could enhance growth of *Mycobacterium lepraemurium* in diffusion chambers. Human embryonic skin cells (HESk) were selected for this investigation, since the human leprosy bacillus, *M. leprae*, characteristically is found in skin tissues of infected individuals. Quantitative studies were conducted on the growth of *M. lepraemurium* in chambers containing HESk cells implanted in the peritoneal cavities of mice and guinea pigs, and incubated in Petri plate monolayer cultures of HESk cells. A second objective of this study was to obtain additional data on the growth of *M. lepraemurium* in cell-free chambers maintained *in vivo* to confirm our previous observation (Rightsel & Wiygul, 1971).

METHODS

Animals. Female mice (strain CFW) weighing 20 to 24 g. were used for maintenance of *Mycobacterium lepraemurium* stocks and as hosts for diffusion chambers. Male guinea pigs weighing 250 to 400 g. were used as hosts for diffusion chambers.

Mycobacterium lepraemurium. The Hawaiian strain of *M. lepraemurium* was maintained by continuous passage in CFW female mice by intraperitoneal injection of approximately 1×10^8 acid-fast bacilli (AFB). Suspensions of bacilli for chamber inoculation were prepared from fatty pads of mice infected 3 to 6 months previously. One g. of fatty pad tissue was ground aseptically in a Ten Broeck tissue grinder (Corning 7727) in 30 ml. of sterile 0.9 % saline. Counts of these suspensions were made using the technique of Shepard & McRae (1968).

Human embryonic skin cells (HESk). Cell lines of human embryonic skin were derived from embryonic tissue obtained from spontaneous abortions during the first trimester of pregnancy. Embryonic skin (2.5 cm.²) was minced into fragments approximately 1 mm.³ which were planted in 900 ml. prescription bottles containing 75 ml. of Basal Medium (Eagle) supplemented with 15 % unfiltered foetal calf serum (FCS) and 10 % beef embryo extract (BEE) and incubated at 37°. The cells were subcultured by trypsinization at 4- to 7-day intervals. Suspensions of HESk cells to be used for Petri plate monolayer cultures were propagated in a modified Chang's 4:5:1 medium (Chang *et al.* 1967), consisting of 40 % FCS, 50 % National Cancer Tissue Culture (NCTC) Medium 109 and 10 % BEE. The cells were planted in 35 × 10 mm. Petri plates (no. 3001, Falcon Plastics, California, U.S.A.) at a concentration of 2×10^6 cells per plate and incubated at 32° in a 5 % CO₂ atmosphere.

Diffusion chambers. The assembly, sterilization, implantation, removal and harvest of chamber contents have been described (Rightsel & Wiygul, 1971).

Zero time control chambers. To determine the zero time AFB control count for each experiment, two chambers were inoculated with a calculated number of organisms, sealed and held in 5° in Hanks's Balanced Salt Solution until all experimental chambers had been processed and placed in the various culture systems. The control chambers then were removed, contents harvested, and the mean zero time AFB count was determined from the chamber yields. This zero time count served as the base from which the growth, generation time and increases of AFB were calculated for each experimental chamber yield.

Killed AFB control chambers. As a part of each experiment a sample of the standardized AFB suspension to be used as inoculum was killed by autoclaving at 121° for 15 min. This autoclaved suspension was inoculated into groups of chambers as a killed AFB control. Volumes of the inoculum for the standardized and killed suspensions were the same.

RESULTS

Diffusion chambers were inoculated with 9.13×10^5 *Mycobacterium lepraemurium* and half also inoculated with 10^6 HESk cells. Four cell-free chambers and four chambers containing HESk cells were collected and harvested after 20, 30, 40 and 50 days' incubation and the yields of *M. lepraemurium* determined (Fig. 1). These yields were highly reproducible as shown by the standard deviations of the mean AFB count per chamber. The significance of *M. lepraemurium* replication was examined by *t*-tests and confirmed by *P* values of less than 0.001, which were obtained when the yield from each culture system was compared with the zero time control count. The zero time control count was 8.50×10^5 AFB, representing 93 % recovery of the original inoculum. The calculated inoculum of the killed AFB control was

7.86×10^5 AFB. Maximum growth was obtained when chambers containing organisms with HESk cells were maintained in mice: a 28.8-fold increase in AFB occurred in 50 days as compared with a 15.4-fold increase in chambers without HESk cells. The yields of AFB from chambers with HESk cells were approximately twice the yields from cell-free chambers at 20, 30, 40 and 50 days. The increase in the growth rate of *M. lepraemurium* in cell-free chambers in mice was proportional to the incubation time, with a 3.2-fold increase in AFB between 20 and 30 days, a 3.6-fold increase between 30 and 40 days, and a 5.2-fold increase between 40 and 50 days. The replication of *M. lepraemurium* in chambers with HESk cells

Fig. 1. Comparison of the mean yields of *Mycobacterium lepraemurium* from diffusion chambers with and without HESk cells in different culture systems. Each bar represents the mean AFB count from replicate chambers with standard deviation (I).

in mice showed a 7.1-fold increase between 20 and 30 days of incubation, a 10.0-fold increase between 30 and 40 days, but only a 6.2-fold increase between 40 and 50 days. After 40 and 50 days' incubation, chambers containing HESk cells maintained in mice showed an approximate 2.5-fold greater increase in AFB than in similar chambers maintained in guinea pigs.

The rate of increase in AFB from chambers without HESk cells maintained in guinea pigs and from chambers maintained in Petri plate cultures were similar throughout the observation period at each harvest time. Additional replicate experiments gave similar results and the small standard deviations in the mean fold increases demonstrate that the diffusion chamber technique provides a system which supports consistent and reproducible growth of *Mycobacterium lepraemurium*.

Growth of *Mycobacterium lepraemurium* in cell-free chambers maintained in 40 mice and 28 guinea pigs showed that the increase in AFB over 50 days was significantly greater in chambers maintained in mice ($P < 0.001$) than in guinea pigs (Fig. 2). The most rapid replication in chambers maintained in mice was between 30 and 50 days, whereas with chambers maintained in guinea pigs the growth rate was almost linear through 50 days of incubation.

Organisms from cell-free chambers maintained in mice for 50 days were harvested and transferred by serial passage to new chambers at a concentration of 9.81×10^5 AFB per chamber and implanted in mice. After 50 days' incubation, these chambers were harvested. The mean increase from eight chambers was 12.6 as compared with a 12.2-fold increase from the original chambers (see Fig. 2), showing that organisms harvested from cell-free chambers and transferred by serial passage to new cell-free chambers continue to replicate at the same growth rate.

Fig. 2. A composite of the mean increases of *Mycobacterium lepraemurium* in cell-free diffusion chambers maintained in mice and guinea pigs. Each point represents the fold increase with standard deviation (I) from 40 chambers maintained in mice and 28 chambers maintained in guinea pigs.

Mycobacterium lepraemurium was recovered after 50 days' incubation from cell-free chambers maintained in mice and guinea pigs, and from chambers containing HESk cells maintained in mice, guinea pigs and Petri plate cultures of these cells. After harvesting and counting the AFB from each of the chamber culture systems, the AFB suspensions were assayed for infectivity in mice. Each suspension was diluted to 1.66×10^5 AFB per ml., and 0.03 ml. was injected subcutaneously into the right hind footpad of each of five mice. After 60 days, the swollen and enlarged footpads were removed, sectioned and stained, and the sections were observed microscopically for the presence of AFB. The sections showed dense clusters of organisms distributed through the tissue cells and localized predominately in and around histiocytes. Comparable results were obtained for each of the chamber maintenance systems. These observations showed that *M. lepraemurium* remained highly infective for mice during 50 days of incubation in each of the diffusion chamber culture systems.

A sample of each harvest suspension also was cultured on blood agar plates, Lowenstein-Jensen slants and in thioglycollate broth, all at 37° , to test for the presence of bacterial contaminants. No growth was observed on the blood agar plates or in the thioglycollate broth cultures after 7 days, or on the Lowenstein-Jensen slants after 30 days.

DISCUSSION

This report demonstrates that the growth of *Mycobacterium lepraemurium* in diffusion chambers maintained in mice, guinea pigs, or Petri plate monolayer cultures was enhanced by HESk cells. This is the first report of significant growth of this organism in a system containing cells derived from a species other than the natural hosts for the organism. This study confirms our previous report on the growth of *M. lepraemurium* in cell-free diffusion chambers maintained in mice and guinea pigs (Rightsel & Wiygul, 1971).

Growth of *Mycobacterium lepraemurium* was significantly greater in chambers with and without cells maintained in mice than in similar chambers maintained in guinea pigs. Maximum growth of the bacilli was a 28·8-fold increase in chambers with HESk cells after 50 days' incubation in mice. This compares favourably with the maximum growth of *M. lepraemurium* obtained previously in chambers containing mouse peritoneal macrophages, which yielded a 30·1-fold increase after 50 days' incubation in mice, and in chambers containing LM mouse fibroblasts which yielded only a 14·2-fold increase (Rightsel & Wiygul, 1971). The shortest generation time of *M. lepraemurium* obtained in chambers with HESk cells maintained in mice was compared to 6·8 days obtained in chambers with macrophages (Rightsel & Wiygul, 1971), or 7·0 days in monolayer cultures of macrophages (Chang *et al.* 1967).

The evidence indicates that *Mycobacterium lepraemurium* may not behave as an obligate intracellular parasite in this particular system since the organism replicates in a cell-free environment within chambers maintained in mice and guinea pigs. Chambers maintained in mice (animals susceptible to infection with *M. lepraemurium*) yielded approximately twice the number of organisms obtained from chambers maintained in guinea pigs which are insusceptible animals. This indicates that the peritoneal fluids of mice apparently contain more of the required growth factors than guinea pig fluids. Guinea pigs also may have effective cellular immune mechanisms which resist infection, such as phagocytic cells which engulf and destroy the organism or otherwise render it incapable of replication. The limited growth of *M. lepraemurium* in cell-free chambers in guinea pigs thus could be due to protection of the organisms from these host cellular defence mechanisms. In contrast, the defence mechanisms of mice apparently do not effectively resist an infection with *M. lepraemurium*.

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