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Attempts to Establish Host Cells for *Mycobactrium leprae in vitro* by Hibridizing Mouse Macrophages and HeLa Cells

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Various primary cells and an established cell line were cultured in roller tubes and in suspension to evaluate their potential roles as host cells to support the growth of *M. leprae in vitro*. The primary cells originated from the organs of chipmunks, mice and humans. Phagocytic ability of those cells except for macrophages was found to be low and did not vary much according to their origin. However, when macrophages from mice peritonial exudate were exposed to the bacteria, the phagocytic efficiency was higher than 47%. Inspite of those good primary results, the macrophages are not cells which can adapt well *in vitro* for long term culture, which is essential for the growth of such a slow growing *M. leprae*. Thus, Somatic cell hybridization between the macrophages and HeLa was made by fusing them with polyethylene glycole. Those hybrids appeared to have both the characteristics of the parent cells, which can provide a natural intracellular environment such as the macrophages and the infinite growth capability of the HeLa cells *in vitro*.

Key Words: Hybridization, Phaqucytosis, Intracellular pavasity cell fusion, Polyethylene glycole.

It was 1878 when *Mycbacterium leprae* (*M. leprae)* was discovered in human leprosy patients and was confirmed as the etiologic agent of the disease by Hansen. Since then, numerous challenges have been made to cultivate the bacteria *in vitro*. Various kinds of cell free culture systems have been developed to support the bacterial growth in artificial conditions. Ogawa's media which contains egg yolk preparation was recently claimed to be a favorable media for the cultivation of the bacteria *in vitro* (Mori, 1974). There are many reports on the observation of enhanced population size of the bacteria by supplementing some materials, i.e., hyaluronic acid(Matsuo, *et al.*, 1975) and cholesterol lecithin (Kato, *et al.*, 1978). An observation of continuous but limitted multiplication of *M. leprae* in colonies was reported when semi-synthetic soft agar were substrated by Murahashi and Yoshida(1973).

Since the bacteria multiply within the host cells in infected humans, it was generally concluded that the bacterial multiplication may re-

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quire certain intracellular factors in host cell cytoplasm. Cell cultures, therefore, were intensively studied for their role as host cells for the bacteria. Garbutt(1965) tried to culture *M. leprae* in human embryonic lung cells(HELU). Samuel *et al.*,(1973) and Drutz(1972) observed multiplication of the bacteria in a limit extent in human macrophage cultures. New-born rat heart cells were also studied and reported as a possible cell which were able to support the bacterial multiplication up to several months (Pattyn, 1974). Cells originating from nerve tissues (Cilia, 1974) and from brain tumor, schwanoma, were employed for the bacterial cultivation(Lalitha, *et al.*, 1979).

Those investigators hoped that nerve cells might be an ideal host cell system since the bacterial infection involves peripheral nervous system in natural human infection. Growth was negative. Nomaguchi (1973) tried and failed to culture the bacteria by use of A31 cells which are a clone of 3T3 cells derived from BALB/C mouse embryo.

Bergal(1973) reported that *M. leprae* multiplied in the necrotic tissue of mouse foot pad and necrobiotic graft of leproma.

Almost the same approaches were made to culture *Mycobacterium lepramurium (M. lepramurium)* which is the etiologic agent for murine leprosy.

Meanwhile, many leprologists extensively searched for a susceptible animal host which can take *M. leparamurium* and/or leprae infection.

Animals such as mice, rats, chipmunks hamsters, and non-human primates were experimented upon. Recently, armadilos were found to be susceptible to *M. leprae* infection. This animals are now widely accepted as a suitable host animal. For the *M. lepramurium*, Shepard (1975) reported that the bacteria can be successfully maintained through serial passages from one mouse footpad to another. However, the results are still controversial.

Although many reports have accumulated claming successful growth of the bacteria *in vitro*, the results are not consistant and hardly reproducible. One of the most important problems in employing an animal cell culture system for the bacterial growth is to find a cell system which satisfy both the conditions, i.e., the cell must provide the optimal conditions for the bacterial survival and the cell itself must survive long enough to meet the slow growth rate of the bacteria.

Almost all of the cells available *in vitro* usually multiply much faster than the inoculated, *M. leprae*, which might lead to dilution phenomena thus interfering in obtaining true value of the bacterial multiplication. The generation time of *M. leprae* is known to be about 10 to 15 days (Garbutt, 1965) and that of the cultured cells are usually less than one day. Attempts to slow down the cell cycle were made by minimizing the metabolic activities of the host cells by incubating them at a subnormal temperature (30°C.) in a serum depleted media (Matsuo, 1973). The bacteria require a lag period of 2 to 4 months to adapt to a new niche before they commit themselves to an active multiplication phase. Therefore, any kind of cell culture system employed for this purpose must be cultured or maintained for a certain period which is to be long enough to meet the bacterial growth phases. The cell cycle of the individual cells also must be long or retarded without losing the original characteristics of the host cell. Furthermore, doubling time of *M. leprae* in a cell culture system appeared to be significantly longer than that observed *in vivo* (Yang and Lew, 1971).

In this study, a cell line which demonstrate highly efficient phagocytic activity upon was selected *M. lepare* among various primary cell lines. This cell line was then fused, with an estlished cell line, HeLa, to obtain a hybrid which

might have both phenotypes of the parent cells. The hybrids would provide the most favorable cells for the bacterial culture.

MATERIALS AND METHODS

Cell preparation and maintenance; Primary cells of lung and kidney from chip munks were obtained by trypsinizing the fresh specimens from the animals. The cells were then expended in roller tubes containing M199 tissue culture media of 20% fetal calf sera(FCS, Microbiological Associate, U.S.A.).

When those cells were confirmed to be adapted to the system in appreciable numbers, which usually took 10 to 20 days post-explantation, the cells were then trypsinized to adjust the number of the cells for the experiments.

An established cell line, HeLa, which grow infinitely *in vitro* was used for the suspension cultures and the hybridization work. Freshly prepared C3H mice peritoneal exudate cells were also prepared by washing the peritoneum of the animal. The macrophages from the peritoneal exudate were maintained in M199 media containing 60% horse sera throughout the experiments.

***M. Leprae* preparation and inoculation;** Fresh *M. leprae* were harvested from biopsied human lepramatous tissue of the patients by trypsinization. The bacteria were then quantitatively adjusted for the inoculation onto the cells in culture. The cells in culture were exposed to bacteria suspended in M199. The number of bacteria and the cells were adjusted to be one multiplicity of inflection(MOI) throughout the experiments. An average of 12 hours for the successful endocytosis by the host cells was allowed at 37°C. in the humidified CO_2(5%) incubator. The HeLa cells were cultured in two methods, one in a flat culture system another in a suspension chamber from the beginning of the co-cultivation with the bacteria.

Somatic cell hybridization between mouse macrophages and HeLa cells; Macrophages from mice periotoneal exudate and HeLa cells were hybridized. An equal number of mice macrophages and isolated HeLa cells by trypsin digestion, 10^7 cells/ml, were concentrated in a centrifuge tube and mixed by gentle shaking. Into this tube, 60% polyethylene glycole (PEG; MW 1000, Ludwick Cancer Inst. Switzerland) were introduced drop by drop keeping the cells in 40°C. water bath for 60 seconds. The PEG treated cells were then dipensed into cell culture vessels containing growth media.

Specific selection procedure for the hybrids was not attempted. However, the cells fused together were easily recognized by locating the cells which had the bacteria in the cytoplasm of the HeLa cells which were not exposed to the bacteria before the fusion. The macrophages are known to be unstable in ordinary culture system and the unfused cells will degenerate within a few weeks.

RESULTS AND DISCUSSION

1) Phagocytic activities of primary fibroblast-like on *M. leprae* Table 1).

Various primary cells explanted in roller tubes and in culture flasks were comparitively analysed for phagocytic ability on the bacteria by their origin and the type of culture. It was found that these primary cells were not the cells of choice for bacterial culture according to their low efficiency of endocytosis(less than 6%). This low efficiency was not influenced by their origin or the culture method. The results could be a reflection of the original host-parasite relationship in nature.

The cells, except the human foreskin cells originated from animals which are not the natural hosts and the organs from which the cells originated were not the sites where the infec-

Table 1. Phagocytic Activity of Various Primary Cells

Origin	Culture system	Replicates	Ave. No. of Phagocytized cells/100
Chipmunk			
Lung	Roller Tubes	3	4.6
Ovary	"	8	3.2
Kidney	"	9	5.8
Human			
Epithelium	"	3	4.7
Mouse			
Macrophage	"	9	47.6

These cells are primary diploids which were inoculated with *M. leprae* at a ratio 1:1.
For the examination of the cells, Acid Fast (AF) staining were employed throughtout the studies.

Table 2. Phagocytic Activity of HeLa Cells in Various Culture System

Culture system	Replicates	Ave. No. of Phagocytized Cells/100
Monolayers	7	17
Suspension	3	21.4

AF stained specimens were examined under 1000X light microscope.

tion take place in the natural human host.

The low efficiency in phagocytic activity of the human foreskin could be explained by the cytoplasmic incompatibility between the non-macrophage and *M. leprae* which is known to parasitize in human macrophages.

2) Phagocytic activities of established cell line, HeLa, (Table 2)

The initial event of phagocytosis is the engulfment of the available materials for cells in culture. When this activity of HeLa cells was compared in their culture conditions, the cells in suspension culture appeard to be slightly higher than that of monolayered cells in the flasks. The overall percentage of the cell's phagcytic activity, regardless of their culture conditions, appeard to be significantly higher than that of the primary non-phagocytic cells tested. This characteristic of HeLa might be expected in almost all of the other established cell lines since the enhanced phagocytic activity is one of the major changes occuring from long term culture *in vitro*. inspite of this moderately high efficiency of phagocytosis of the HeLa cells, the validity of the cells as a host for *M. leprae* has not been proved by many previous workers. One advantage of employing this cell for the bacterial culture might be the characteristic of infinite growth *in vitro*. Although the cell cycle is considerably faster than that of the normal unestablished cells, it is almost impossible to select a cell line of slow growth unless it is artificially retarded.

3) Phagocytic activities of professional cells, mouse peritoneal macrophages (Table 1)

Among the cell lines tested, macrophages from mouse peritoneum were found to be the best cell system for phagocytic ability upon the bacteria(more than 47%). The macrophages are the cells claimed to be the natural hosts for *M. leprae* in human patients. The survival of the bacteria *in vivo* could be explained by the continuous influx of new macrophages into the site of

bacterial multiplication, which might overcome the insufficiency in supporting the bacterial multiplication by the cells due to their relatively short life span. The phagocytosis of these cells could be the normal behavior as scavanger cells in the first line of defence in infection. However, it is also possible that the macrophages provide a favorable niche for the intracellular parasites such as *M. lepare* which evolved in adapting themselves to that environment without being digested by the cells. For the *in vitro* culture system, a continuous input of fresh macrophages into the *M. leprae* infected macrophages would be necessary to mimic the situation *in vivo*. However, this will not be practical because of the difficulties in maintaining those systems for a long time and the genetic incompatabilities between the old cells and newly introduced cells which may be heterogenous in terms of their origin and the adaptability to *in vitro* en vironment. Failure on this aspect was reported also by other workers. Therefore, it might be reasonable to change those cells into a type of cells which can survive for a long time without losing their original characteristics.

4) Characteristics of hybrids between macrophages and HeLa cells (Table 3)

The ideal hybrid would have the characteristics of macrophages which are known to be a natural host and have a high efficiency of phagocytosis and the HeLa cells which grow infinitely *in vitro*. In the production of a somatic cell hybrid the first step is cell fusion. Using agent such as inactivated Sendai virus or Polyethylene glycole (PEG) (Pontecorvo, G. (1975)) a high proportion of cells can be induced to fuse. In this study, PEG was used for the cell fusion and the frequency of cytoplasmic fusion were relatively high when normal HeLa cells were fused with bacteria engulfed macrophages (average 80%). The average phagocytic efficiency of the fused cells was relatively high which was quite comparable with that of unfused macrophages (32.0%). The most important observation in any kind of experiment on the attempts to culture a certain bacteria is of course the true value of their perpetuation in the system. Although these experiments were continued for too short a period to draw any possible conclusive results, it was clearly shown that the hybrids have the original characteristics in terms of phagocytic behavior and extended growth capability *in vitro* for a long time at least beyond the life span of the normal macrophage. The number of bacteria internalized inside the hybrids appeared to be increased by more than twice within the 24 days of post inoculation (Table 4 and Fig. 1). It is generally accepted that only a small fraction of the fused cells will undergo the next step, nuclear fusion, and give rise to a viable hybrid cell line. Therefore it is recommanded that a stable hybrid

Table 3. Phagocytic Activity of Hybrids (C_3H Mϕ x HeLa)

Cells	Replicate	Average No. of cells phagocytized
C_3H Mϕ	9	47.6
HeLa	7	17
C_3H x HeLa	5	32

AF stained, under 1000X light microscope

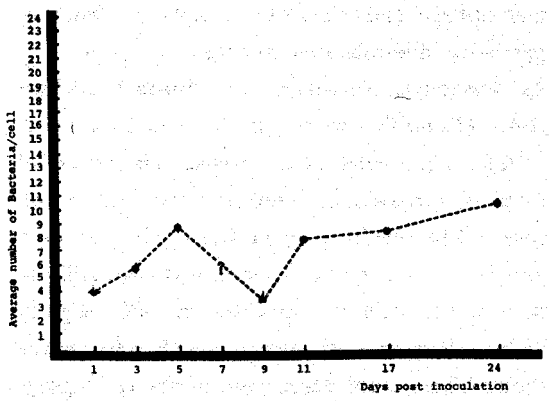

Fig. 1 Changes in Number of Bacteria in HeLa Cells.

Table 4. Changes in Number of Bacteria in HeLa Cells

	Days post inoculation							
	1	3	5	7	9	11	17	24
Ave. No. of Bacteria /cell	4.0 (4-10)	5.8 (4-10)	8.8 (4-15)	?	3.55 (1-9)	7.85 (3-25)	8.1 (3-25)	10.25 (4-22)

One hundred cell groups with bacteria in the cytoplasm were counted for the calculation of the average number of the bacteria. The specimens were stained by Acid fast method and examined under light microscope (1000X oil emersion)

cell line should be made before employing this culture system for cultures. The stability of fused cells between cells of different species is known to be unfavorable for the long term culture and the fate of any kind of cells fused with phagocytic cells is still unanswered.

CONCLUSION

Among the cells tested in this experiment, the hybrids between mouse macrophages and HeLa cells were found to be the best cellular hosts for *M. leprae* in their cytoplasm. Therefore, its tentatively concluded that the hybrids could be the cells of choice for further investigation in research works attempting *in vitro* culture of the bacteria because the hybrids still maintains fairly good phagocytic activity on the bacteria, infinite growth characteristics *in vitro* and the possibility of culture *in vivo* when the parent cells originate from inbred strains of experimental animals.

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