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Mycobacterium Leprae in Cultured Mouse Peritoneal Macrophages: In vivo Infection In vitro Cultivation

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ABSTRACT

To grow Mycobacterium leprae in cultured mouse peritoneal macrophages, studies were made on 1) the purification of M. leprae from lepromatous nodules by trypsinization, 2) growth experiment of purified M. leprae in cultured macrophages by in vivo infection-in vitro cultivation technique and 3) the observation of pathological changes in spleens of mice induced by intraperitoneal inoculation of purified M. leprae. Results are summarized as follows.

1. A simple and effective procedure is described for purification of M.leprae from biopsied nodules of lepromatous leprosy patients by trypsinization and high speed centrifugation. The procedure resulted in a good yield of homogeneous preparation of M. leprae with a negligible contamination of tissue debris.

2. Significant decreases were observed in the numbers of acid-fast bacilli in cultured macrophages and of macrophages harboring acid-fast bacilli by the length of intervals between the time of intraperitoneal inoculation of purified M. leprae and the time of initiation of macrophage cultures.

3. Microscopic examination of stained preparations of macrophages cultured by in vivo infection-in vitro cultivation technique indicated that an apparent increase in the number of acid-fast bacilli in the macrophages occurred when the cultures made at 24 hours and 1 week after inoculation were maintained in vitro up to 2 months or more.

4. Pathological changes in the spleens of mice inoculated with purified M. leprae were of mainly degenerative nature in the red pulp. No multiplication of M. leprae was observed in the spleens of mice up to 5 months after inoculation.

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INTRODUCTION

Following Shepard's observations(1960 a, 1960 b) that a limited but consistent growth of Mycobacterium leprae occurred in the foot pad of mice, a large amount of experimental works on animal transmission of M. leprae has been made. It is now widely accepted that M. leprae can be grown in a variety of rodents including mice.

Since the growth characteristics of M. leprae, ie, 1) failure of growth on bacteriological lifeless media, and 2) nature of obligate intracellular growth resembled closely to those of the viruses, numerous attempts have been made to cultivate M. leprae in tissue

culture cells. However, owing to the unusually long generation time of M. leprae (Shepard and McRae 1965, Garbutt 1965) many of the experimental works on the growth of M. leprae in tissue culture system have provided negative results.

As a experimental model for the growth of M. leprae in tissue culture, mouse peritoneal macrophage has been used for in vitro cultivation of M. lepraemurium (Chang and Neikirk 1965, Lee et al. 1967, Yang and Lew 1968), and quantitative evidences for the actual growth of M. lepraemurium in cultured mouse peritoneal macrophage have been reported(Chang et al. 1967, Yang and Lew 1968).

Among the experiments of the cultivation of M. leprae in tissue culture system, Chang and Neikirk(1965) observed some features of growth of M. leprae in cultured mouse peritoneal macrophages, and Garbutt(1965) reported the growth of M. leprae in cell lines of 14 pf rat fibroblast and human embryo lung. Nevertheless, exact biological characteristics of so-called tissue culture grown M. leprae has not been fully elucidated.

As a preliminary for the cultivation of M. leprae in tissue culture cells, studies were made on 1) the preparation of trypsin-purified M. leprae from biopsied nodules of lepromatous leprosy patients, 2) growth experiment of trypsin-purified M. leprae in cultured mouse peritoneal macrophages by in vivo infection-in vitro cultivation technique and 3) pathological changes in the spleens of mice inoculated with trypsin-purified M. leprae.

MATERIALS AND METHODS

1. Trypsin purification of M. leprae from lepromatous nodules:

During the work on tissue culture of trypsin-dispersed cells from the spleens of mice infected with M. lepraemurium, it was observed that prolonged trypsinization (0.25% trypsin in PBS) of minced spleen tissues at 37°C resulted in significantly low yield of cultivable macrophages(or macrophage-like cells), and that the supernatant from the trypsinization mixture contained a large amount of freely released M. lepraemurium (Yang and Lew 1971). Based on this observation, trypsin purification of M. leprae from the biopsied lepromatous nodules was carried out by the procedure indicated in Fig. 1.

The biopsied nodules were obtained from lepromatous leprosy patients at the World Vision Special Skin Clinic, Seoul, Korea (Table 1).

The nodules were processed for trypsin purification of M. leprae either immediately

Table 1. Biopsied nodules from lepromatous leprosy patients

OPD No.*	Name	Sex	Age	Skin bacteriology (Wade's method) BI #1	SFG #2	Date of biopsy
4837	S.B. Ahn	M	35	5+	0—2—0	Jan. 1971
4901	K.H. Cho	M	34	5+	0—2—0	May 1971
4913	S.J. Kim	F	17	4+	0—2—0	Jun. 1971

*: OPD No. at the World Vision Special Skin Clinic, Seoul.
#1: Bacterial index(Ridley 1964 a)
#2: SFG value(Ridley 1964 b)

after the biopsy or kept frozen at—15°C before use(Fig.1).

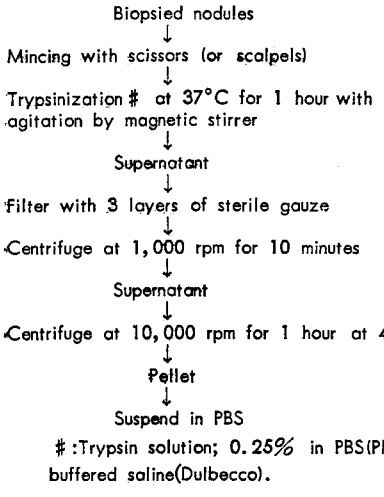

Fig. 1. Trypsin purification of M. leprae from biopsied lepromatous nodules.

2. In vivo infection- in vitro cultivation technique:

One-tenth milliliter of PBS suspension of purified M. leprae was injected intraperitoneall yinto adult mice of a random bred CFW strain($1.6\sim4.6\times10^7$ bacilli/mouse), and the cultures of peritoneal macrophages were made at certain intervals after inoculation by the procedure reported previously(Yang and Lew 1968). For each culture experiment, 4 to 5 mice were used.

The numbers of macrophages in peritoneal washings were approximately 1.0×10^6 cells/ml. The cultures of macrophages were made by inoculating 1 ml of peritoneal washings into a glass Leighton tube or plastic petri dish(35×10mm Style, Falcon Plastics) and incubating at 34°C under 5% CO_2 atomsphere.

Growth medium for macrophage culture consisted of double strength NCTC 135; 5 parts, heat inactivated bovine serum; 4 parts and diluted(1:5) bovine embryo extract; 1 part. The medium was changed at 3 to 4 day intervals. Only penicillin was added to PBS, trypsin solution and NCTC 135 at the concentration of 200 to 300 units/ml.

3. Enumeration of numbers of acid-fast bacilli (AFB):

The numbers of AFB both in PBS suspension of trypsin-purified M. leprae and in the ultrasonicate of cultured macrophages in Leighton tube or plastic dish were determi ned by the pin head method of Hanks (1968).

For ultrasonic disruption of cultured macrophage in a tube or dish, old meidum was decanted and 1.5ml of prewarmed(37°C.) trypsin solution was added. Then, the macrophages were detached from glass or plastic surface with rebber police and the cell suspension was treated by ultrasonic disintergrator(Ultrasonic Probe, Fisher Model BP-2) at 20 KC for 4 minutes.

4. Microscopic observation of acid-fast stained preparations of cultured macrophages:

After the initiation of macrophage cultures by in vivo infecion-in vitro cultivation technique, cover slip in the Leighton tube was stained for AFB(Yang et al. 1968) and examined microscopically to follow the changes of M. leprae inside cultured macrophages.

5. Pathological findings in the spleens of mice inoculated intraperitoneally with trypsin-purified M. leprae:

At the time of culture of peritoneal macrophages by in vivo infection-in vitro cultivation technique, spleens were removed and hematoxylin-eosin, and AF stained preparations were made. Pathological findings observed in the spleens of mice inoculated with trypsin-purified M. leprae were compared with those of mice infected with M.

lepraemurium intraperitoneally.

RESULTS

1. Preparation of trypsin-purified M. leprae:

As shown in Fig. 2 and Fig. 3, trypsin purification of M. leprae from lepromatous nodules resulted in good yield of singly well-dispersed population of M. leprae with a negligible contamination of tissue debris. The supernatant obtained after centrifugation at 10,000 rmp for 1 hour at 4°C(see Fig.1.) contained only a scanty number of AFB. This method allows to prepare a highly concentrated suspension of purified M. leprae by adjusting the volume of suspending PBS to the pellet obtained by centrifugation at 10,000 rpm for 1 hour.

2. Changes in the numbers of AFB inside cultured macrophages in relation to the time of tissue cultures after intraperitoneal inoculation of purified M. leprae into mice:

About 30 mice were inoculated with purified M. leprae, and tissue culture of peritoneal macrophages from 4 to 5 mice were made at 24 hours, 1 week, 4 weeks, 6 weeks, and 5 months after the inoculation. As shown in Fig. 4, 5 and 6, a marked difference was observed in the numbers of AFB per macrophage and of macrophages harboring AFB between the cultures made at different times after the inoculation. Significant decreases in the numbers of AFB per cultured macrophage and of macrophage harboring AFB were apparent in the cultures made at 4 and 6 weeks and 5 months.

In support of these microscopic findings of stained preparations of cultured macrophages, enumeration of a total number of AFB per macrophage culture following ultrasonic treatment of the cells demonstrated sharp decreases in the numbers of AFB in macrophages by the time of tissue culture after intraperitoneal inoculation of purified M. leprae as shown in Table 2.

Table 2. AFB counting of macrophage cultures after ultrasonic treatment

Time of tissue culture made after inoculation	Total number of AFB/plate #1
24 hours	1.8×10^5*
	9.2×10^4
	2.8×10^5
1 week	9.2×10^4
	1.4×10^5
	none #2
4 weeks	none
	none
	none
6 weeks	5.4×10^4
	none
	none
5 months	none
	none
	none

#1: Plastic petri dish, 35mm diameter.
#2: Minimum number of AFB countable by the pin head method $=4.0\times10^4$
*: The number represents a total number of AFB from each of 3 plates randomly selected from the cultures.

The numbers of AFB in macrophage cultures made at 4 and 6 weeks and 5 months after inoculation were all below the level of 4.0×10^4 that is the minimum number of AFB countable by the pin head method in this system, except 1 plate among the 6 weeks culture. It became clear that the longer the state of in vivo infection was, the smaller were the numbers of AFB in the cultured macrophages countable at the time of tissue culture, and that there was no indication of intracellular growth of M. leprae inside peritoneal macrophages in vivo up to 5 months following intraperitoneal inoculation.

However, when the macrophage cultures

made both at 24 hours and 1 week after intraperitoneal inoculation of purified M. leprae were maintained in vitro at 34°C under 5% CO_2 atmosphere for a prolonged period of time, the features of significant increases in the number of AFB inside individual macrophages became evident(Fig. 7a ~7f). At the same time most of the cultured macrophages exhibited the morphological alteration of activated macrophages.

3. Pathological findings of the spleens of mice inoculated with trypsin-purified M. leprae:

Macroscopically, some of the spleens appeared to be considerably enlarged when examined at the time of macrophage cultures after intraperitoneal inoculation. Microscopic observation revealed characteristic changes as shown in Fig. 8. A predominant pathologic involvement was the wide-spread hyalinization in the red pulp of the organ. There was a slight indication of lymphoid hyperplasia and extramedularly hematopoietic activity in the white pulp, but in general the white pulp appeared to be shrunken.

The earliest of these changes were manifest even 1 week after intraperitoneal inoculation. However, no multiplication of M. leprae was observed in the spleens up to 5 months after inoculation.

DISCUSSIONS

1. Trypsin purification of M. leprae from lepromatous nodules:

The aims of the trypsin-purification of M. leprae from lepromatous nodules are 1) the collection of viable, infectious M. leprae, 2) the avoidance of mechanical damages to M. leprae given by the conventional collection procedures such as grinding in mortar and pestle, and homogenizer treatment and 3) the removal of contaminating tissue debris from M. leprae preparations.

Our purification method resulted in the collection of sterile purified M. leprae from minced tissues of lepromatous nodules. An unique feature of this procedure is the complete omission of either grinding or homogenization of lepromatous tissues which became a routine in the procedures of purification attempts by others.

Trypsin(Lew and Carpenter 1956, Nakamura 1956, Nakamura and Ueno 1963) and chemicals(Hanks 1951, Nishimura 1961, Nakamura and Ueno 1963, Mori et al. 1961) have been used for the purification of M. leprae and M. lepraemurium from the infected tissues. In our study trypsinization of minced lepromatous nodules was carried out with 0.25% trypsin in PBS for 1 hour at 37°C with agitation by magnetic stirrer. The effect of such a purification of M. leprae by trypsin treatment on the viability (infectiousness) of M. leprae are now being tested by the foot pad inoculation of purified M. leprae into mice and other animals. However, following evidences support the assumption that the preparation of trypsin-purified M. leprae is viable and infectious, ie, 1) Lew and Carpenter(1956) observed that trypsinization of unboiled M. lepraemurium-infected tissue with 0.5% trypsin for 90 minutes at 37°C did not result in the loss of viability of M. lepraemurium and 2) Nakamura and Ueno(1963) reported that trypsin(0.25% in final concentration) treatment of infected tissue for 60 minutes at 37°C for the isolation and purification of M. lepraemurium fully retained the infectivity of the bacilli.

2. The in vivo infection-in vitro cultivation experiment of M. leprae in mouse peritoneal macrophages:

By this technique excellent growth of M. leprae-infected macrophages were obtained in tissue culture, and the cultures could be maintained in viable, good condition up to 3 months after the initiation of tissue culture without much difficulty. It appeared that the use of glass tubes was far superior to plastic plates for a prolonged maintenance of cultured macrophages in vitro.

Recently, many studies have been made on the activation of mouse peritoneal macrophages by infection with certain microbial agents and others (Mallucci 1969, Gordon and Cohn 1970, 1971, Virolainen and Defendi 1967, North 1969 a, 1969 b, Mackaeness and Blanden 1967, Blanden et al. 1969), and Godal et al. (1970) observed macrophage activation when the cultures of blood-derived macrophages from the tuberculoid leprosy patients were exposed to M. leprae in vitro in the presence of lymphocytes.

The appearances of the cultured macrophages(Fig. 7a~7f) showed morphological alterations similar to those observed in the activated macrophages(Blanden 1968, Blanden et al. 1969). However, no study has been made whether the infection of mouse peritoneal macrophages with M. leprae could induce per se activation of the macrophages or not.

The numbers of AFB per macrophage and of macrophage harboring AFB declined sharply depending on the interval between the time of intraperitoneal inoculation of purified M. leprae and the time of macrophage tissue culture. Quantitative assessment of the numbers of AFB in the macrophages at the time of tissue cultures(Table 2) corresponded well to the microscopic findings of the stained preparations of cultured macrophages(Fig.4 5 and 6). Sharp decreases in the numbers of AFB in the macrophage cultures made at or after 4 weeks may reflect the dilution effect by the turn-over of macrophage population in the mouse peritoneal cavity, since the peritoneal macrophages are considered end cells, derived from circulating monocytes, in a normal, steady state situation(van Furth and Cohn 1968), and turn-over rate of peritoneal macrophage is estimated at about 0.1% per hour and turn-over time(Trasher 1966) can be estimated at about 40 days(van Furth and Cohn 1968).

When the macrophage cultures made at 24 hours and 1 week after inoculation were maintained in vitro up to 2 months, a sign of increase in the number of AFB inside individual macrophage became gradually apparent in stained preparations(Fig.7a~7f).

Earlier experiments to assess the quantitative increases in the numbers of AFB in cultured macrophages by ultrasonic treatment failed because of the limited period of maintenance of macrophage culture in the plastic petri dishes. The cultured macrophages can be maintained in glass Leighton tubes for much longer periods of time in a good viable condition than in the plastic dishes. Experiments are now in progress to follow the changes in the total number of AFB in cultured macrophages maintained in glass Leighton tubes.

Garbutt(1965) reported multiplications of M. leprae in the cultures of cell lines of human embryo lung and 14 pf rat fibroblast by serial transfer technique, and pointed out the importance of human element-either human cells grown in medium containing non-human serum or rat cells grown in human cord serum. It is planned to determine the effect of the human element on the multiplication M. leprae in our system.

The phenomenon of a lag phase has been observed in the experimental inoculation of M. leprae into the foot pad of mice and other rodents(Hilson 1965, Rees 1965, Rees and Weddell 1970, Shepard and McRae 1965, Yang and Lew 1971), and Garbutt(1965) demonstrated a lag phase of approximately 3 months before M. leprae adapted to a new environment(tissue culture cells) and began to multiply actively. The presence of such a lag phase may become a limiting factor in experiments to grow M. leprae in the mouse peritoneal macrophage system.

The exact life span of mononulcear phagocytes either in the tissues or in vitro remains to be measured. Nevertheless, an unusually long life span of mouse peritoneal macrophages in vivo was indicated by the demonstration of van Furth and Cohn(1968) that about 1% of the mouse peritoneal macrophages were still labeled 5 weeks after 4 pulses of tritiated thymidine, and after 8 weeks an occasional positive peritoneal macrophage was found. By in vitro cultivation Chang(1964) succeeded in the extended maintenance of cultured mouse peritoneal macrophages up to 220 days, averaging 172. 5 days, and eventually observed the growth of M. lepraemurium in the cultured macrophages(Chang and Neikirk 1965, Chang et al. 1967).

The effects of infection of mouse peritoneal macrophages with M. leprae(or with M. lepraemurium) on the life span of the macrophages are unknown.

3. Pathological changes in spleens of mice inoculated with purified M. leprae:

Unexpectedly, the pathological changes observed in the splesns of mice inoculated with purified M. leprae were of a degenerative nature, involving mainly the red pulp of the organ. No. sign was observed of extensive proliferation of the macrophage (or macrophage-like cells) nor any indication of active multiplication of M. leprae in the organ. The features of pathological involvement in the spleens of mice inoculated intraperitoneally with purified M. leprae differed markedly from those of mice inoculated with M. lepraemurium. These findings may be regarded as one of the important biological distinctions of M. leprae from M. lepraemurium in the mouse system.

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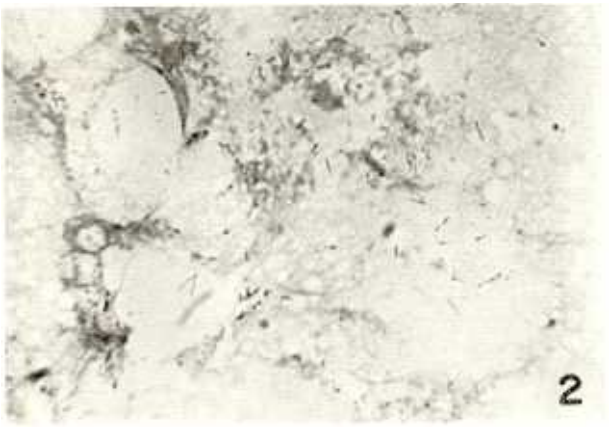

Fig. 2. Bacillary suspension obtained after trypsinization at 37°C for 1 hour of minced lepromatous tissues.

Fig. 3. Final product of trypsin purification of M. leprae from the lepromatous tissues.

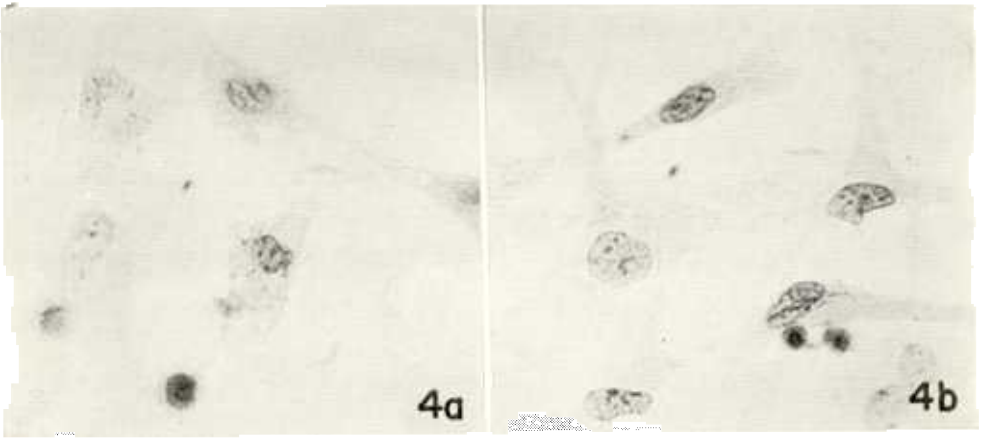

Fig. 4—a & Fig. 4—b. Intracellular AFB observed in the macrophage cultures made 24 hrs after intraperitoneal inoculation of trypsin-purified M. leprae into mice.

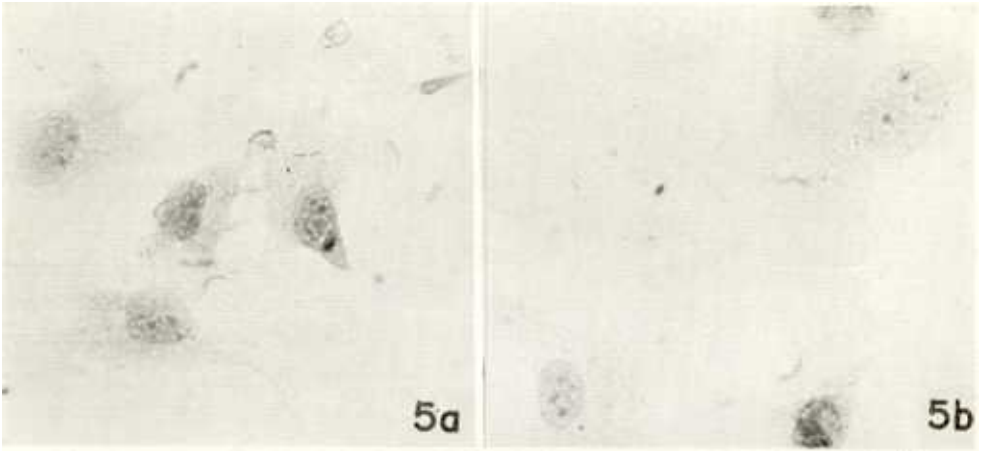

Fig. 5—a & Fig. 5—b. Intracellular AFB observed in the macrophage cultures made 4 weeks after intraperitoneal inoculation of trypsin-purified M. leprae into mice. Notice the apparent decrease in the number of both intracellular AFB and of the macrophages harboring AFB.

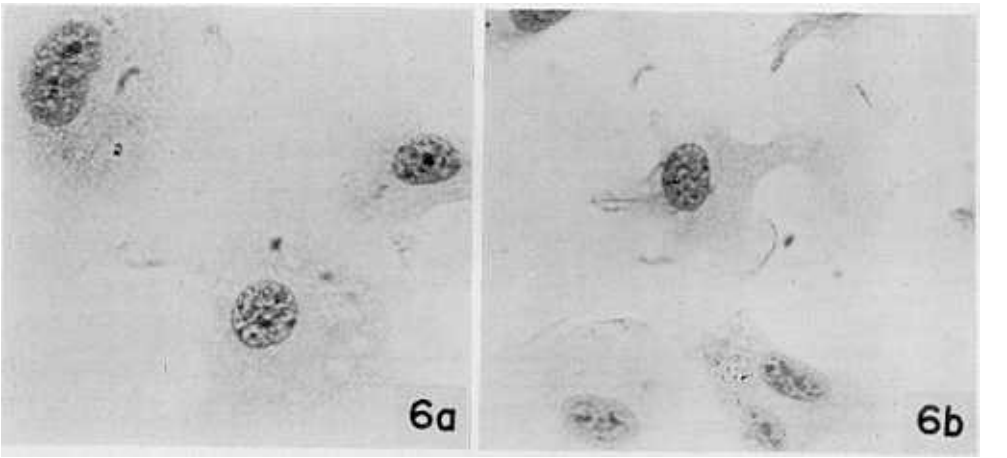

Fig. 6—a & Fig. 6—b. Macrophage cultures made 5 months after intraperitoneal inoculation of trypsin-purified M. leprae into mice.

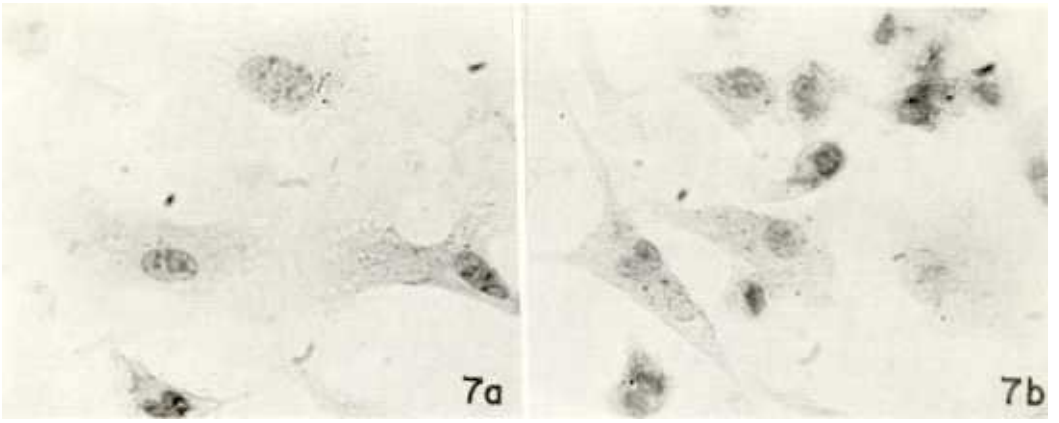

Fig. 7—a & Fig. 7—b. Significant increase in the number of AFB inside macrophages of which culture was made 24 hrs after intraperitoneal inoculation of trypsin-purified M. leprae into mice and maintained in vitro for 34 days.

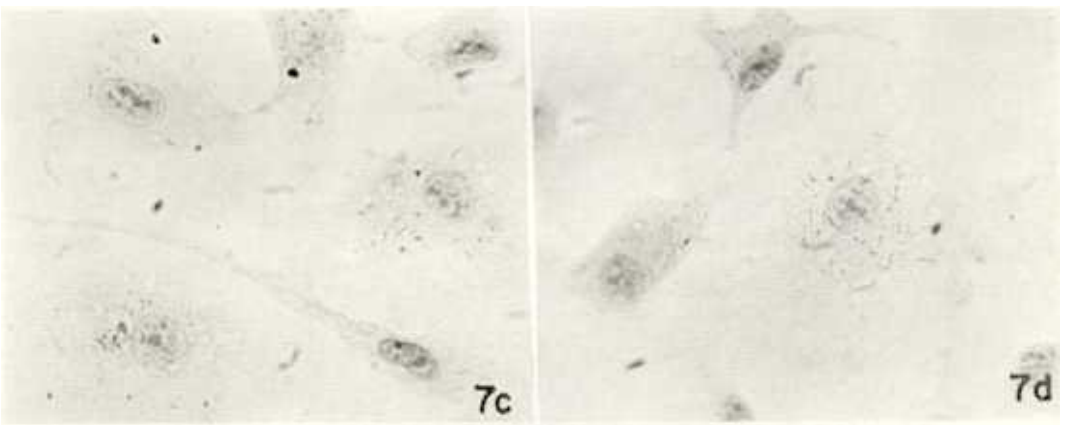

Fig. 7—c & Fig. 7—d. Same culture as in Fig. 7—a and 7—b and maintained for 49 days.

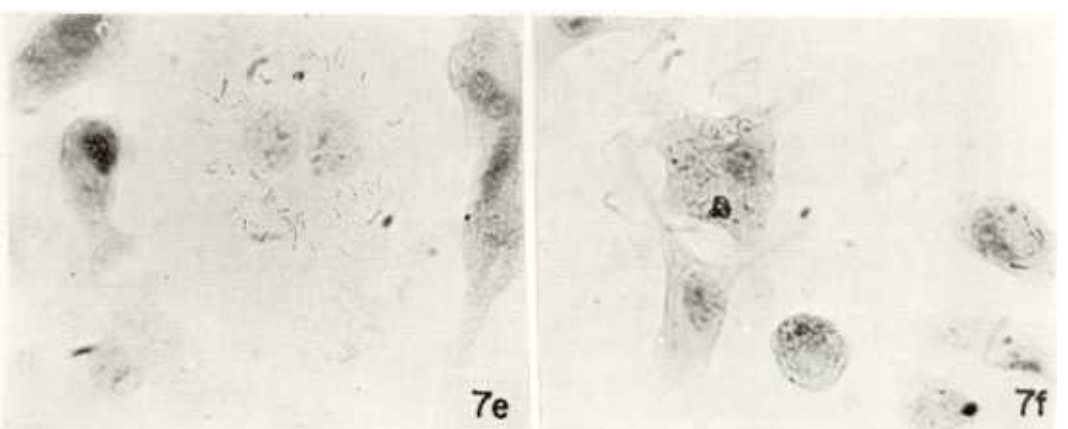

Fig. 7—e & Fig. 7—f. Same culture as in Fig. 7—a and Fig. 7—b, and maintained for 60 days.

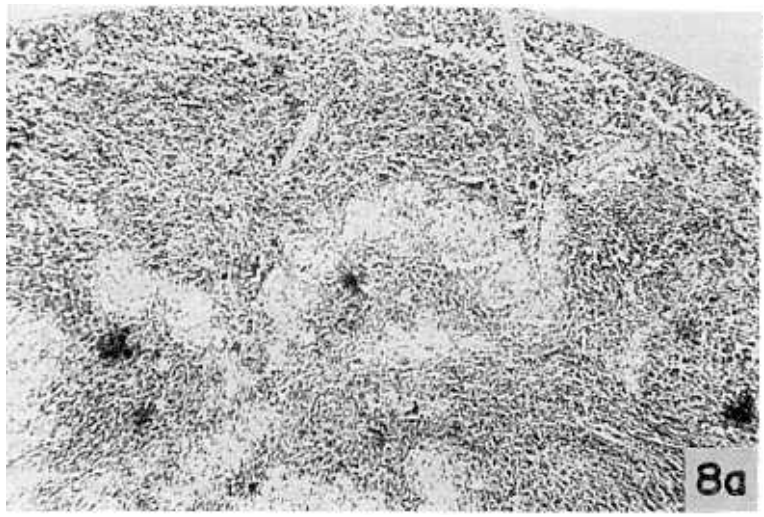

Fig. 8—a. Pathological changes in the spleen of mouse 5 months after intraperitoneal inoculation of trypsin-purified M. leprae.

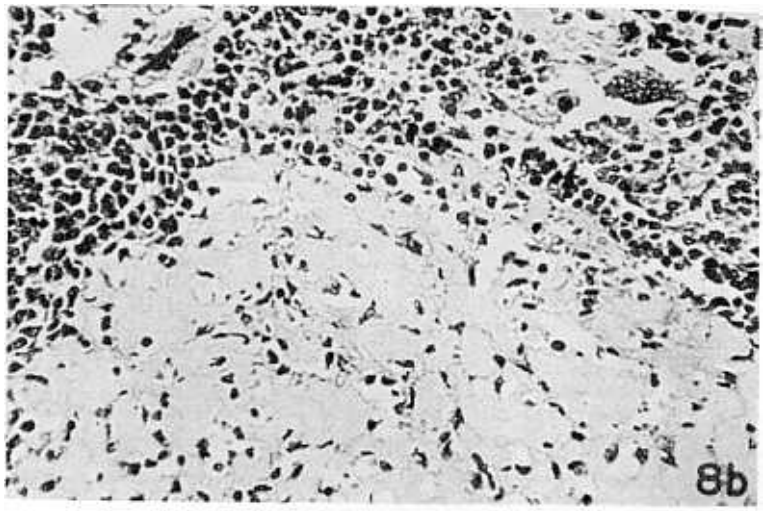

Fig. 8—b. Higher magnification of Fig. 8a,indicating characteristic hyalinization feature in the red pulp of the spleen.