大韓微生物學會誌 第7卷 第1號 1972
J. Kor. Soc. Microbiol. Vol. 7, No. 1, 1972

Growth Experiment of Mycobacterium Leprae in Cultured Mouse Peritoneal Macrophages*

1. Purification of viable Myco. leprae from biopsied lepromatous nodules by trypsinization method.

Yang, Y.T., Lew, J., Cho, S.H. and Kim, J.K.

Department of Microbiology, Yonsei University College of Medicine
Seoul, Korea

—圖文抄錄—

組織培養된 마우스腹腔巨喰細胞에서의 人癩菌增殖實驗*

1. 癩結節에서 trypsin 處理에 依한 人癩菌의 精製

延世大學校 醫科大學 微生物學敎室

梁容泰·柳 駿·趙世勳·金駿杰

生檢한 癩結節에서 簡單하고 效果的인 方法인 trypsin 處理와 高速度遠沈에 依하여 動物接種目的에 使用할수 있는 人癩菌의 精製에 對하여 記述하였다.
本 方法의 한 特徵은 그 精製過程中에 在來式인 癩結節組織의 磨碎나 乳液化操作을 전혀 包含시키지 않은 點이다. 이 方法에 依하여 精製된 人癩菌을 韓國産 다람쥐(Tamias sibiricus asiaticus, Gmelin)의 足底部 및 耳朶組織內에 接種하였던 바 菌接種 8 및 12個月에 이르러 菌增殖樣狀이 惹起됨이 觀察되었다.

ABSTRACT

A simple and effective procedure is described for semi-purification of viable Myco. leprae from biopsied lepromatous nodules by trypsinization and high-speed centrifugation. An unique characteristic of this method is a complete omission of conventional grinding or homogenization of minced lepromatous tissues prior to purification.

Inoculation of trypsin-purified preparation of Myco. leprae into foot pads and ear lobes of Korean chipmunk (Tamias sibiricus asiaticus, Gmelin) resulted in apparent increases in total number of acid-fast bacilli per inoculated tissue 8 and 12 months after inoculation.

INTRODUCTION

In order to attain either concentration or purification of Myco. leprae and Myco. lepraemurium for a particular type of research, a variety of methods have been developed,

* This investigation was supported by financial aids from the World Vision Special Skin Clinic, Seoul and from the Faculty Research Funds, Yonsei University College of Medicine, Seoul, Korea.

namely, repeated centrifugation(Dharmendra, 1941 a[1]) and gradient centrifugation (Hanks, 1951[2]; Kwapinski et al., 1972[3]) as physical means: chloroform (Dharmendra, 1941 b[4]; Dharmendra and Mukerjee, 1952[5]), petroleum ether (Khanolkar and Rajalashmi, 1952[6]), toluol (Lew, 1943[7]), diethyl ether (Lew, 1955[8]), sodium deoxycholate (Nakamura and Ueno, 1963[9]; Nishimura et al., 1961[10]) and chloroform and toluol (Nakamura, 1956 a[11], 1956 b[12]) as chemicals: digestive ferment (Alvarez, 1897[13]) and trypsin (Lew, 1955[8]; Lew and Carpenter, 1956[14]; Kim and Lew, 1972[15]) as proteolytic enzymes: and a number of combined methods (Nakamura, 1956 a[11], 1956 b[12]; Nakamura and Ueno, 1963[9]; Mori et al., 1961[16]; Nishimura et al., 1961[10]).

Little emphasis has been placed on the effect of such methods of either concentration or purification on the viability of Myco. leprae and Myco. lepraemurium except few reports (Lew, 1955[8]; Lew and Carpenter, 1956[14]; Nakamura, 1956 b[12]; Nakamura and Ueno, 1963[9]; Ito and Sonoda, 1957[17]).

Furthermore, all of such concentration and purification methods routinely included rather extensive grinding in motar and pestle or homogenization of minced tissues prior to further procedures. Since both grinding and homogenization techniques have become a laboratory routine to break up bacterial cells for research purposes, it seemed highly probable that either grinding or homogenization technique would exert considerable detrimental effects on the viability of the organisms involved.

Shepard's (1960 a[18], 1960 b[19]) observation that inoculation of Myco. leprae into foot pads of mice resulted in a consistent but limited growth of the organism has been amply confirmed and extended (Rees, 1965[20]; Rees and Weddell, 1970[21]). In addition to the mouse system, a similar pattern of growth of Myco. leprae has been observed in numbers of other rodents (Waters and Niven, 1965[22]; Hilson, 1965[23]). Recently, reports on the growth of Myco. leprae in the foot pads and ear lobes of chipmunk have been made from this laboratory (Lew et al., 1970[24]; Yang and Lew, 1971[25]) and other's (Nakamura and Hisai, 1970[26]).

During the work on tissue cultures of trypsin-dispersed cells from the spleens of mice infected with Myco. lepraemurium, it was observed that prolonged trypsinization at 37°C (with 0.25% trypsin in PBS) of minced spleen tissues resulted in significantly low yields of cultivable macrophages (or macrophage-like cells), and that the supernatants from such trypsinization mixtures contained a large number of freely released Myco. lepraemurium (Chang et al., 1972[27]). Based on this observation, trypsin purification of Myco. leprae from the biopsied lepromatous nodules was carried out in this study.

As a preliminary for the cultivation of Myco. leprae in cultured mouse peritoneal macrophages, the viability of trypsin-purified Myco. leprae was tested by inoculating into foot pads and ear lobes of Korean chipmunks (Tamias sibiricus asiaticus, Gmelin).

MATERIALS AND METHODS

1. Lepromatous nodules:

Lepromatous nodules were biopsied from untreated lepromatous leprosy patient at the World Vision Special Skin Clinic, Seoul, Korea (Table 1). The biopsied nodules were kept frozen at-15°C overnight before trypsin purification of Myco. leprae.

2. Trypsin purification of Myco. leprae from biopsied lepromatous nodules:

Frozen nodules were quickly thawed in 37°C water bath and then processed for trypsin purification of Myco. leprae as indicated in Fig. 1.

Table 1. Biopsied nodules.

OPD No.	Type of Disease	Sex	Age	Skin Bacteriology		Date of biopsy
				BI**	SFG***	
4881	L*	M	21	6+	0—2—0	April, 1971

*;Lepromatous leprosy
**;Bacterial index (Ridley, 1964 a[28])
***;SFG value (Ridley, 1964 b[29])

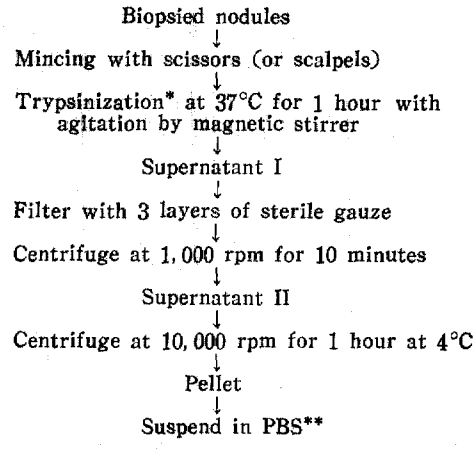

Fig. 1. Trypsin purification of Myco. leprae from biopsied lepromatous nodules.
*;Trypsin solution;0.25% in PBS
**;PBS (Phosphate buffered saline, Dulbecco)

It may be noted that this method of trypsin purification did completely omit grinding or homogenization procedures of minced lepromatous nodules prior to purification procedure.

Residues remaining after trypsinization of minced lepromatous tissues at 37°C for 1 hour could be used for the second cycle of purification.

3. Inoculation of trypsin-purified Myco. leprae into Korean chipmunks:

Korean chipmunks(Tamias sibiricus asiaticus, Gmelin) of both sex, age of less than one year and weighing 50 to 70 gm were used for viability test of trypsin-purified Myco. leprae. A detailed report on cares and handling of the chipmunks for laboratory use and the results of inoculation with Myco. leprae will be published elsewhere. Temperature of animal room was maintained at 20±2°C throughout the experimental period.

One-twentieth ml of diluted suspension of Myco. leprae was inoculated subcutaneously into either left foot pad or left ear lobe of a chipmunk. The inoculum contained approximately 1.0×10^5 of acid-fast bacilli (AFB). The heat-killed (70°C for 1 hour) preparation of trypsin-purified Myco. leprae was inoculated into either right foot pad or right ear lobe and served as control. Two to 4 chipmunks were sacrificed 2 weeks, 8 and 12 months after inoculation and total number of acid-fast bacilli per either food pad or ear lobe was counted by the pin head method of Hanks (1968[30]).

RESULTS

1. Preparation of trypsin-purified Myco. leprae:

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

Table 2. Results of inoculation of trypsin-purified Myco. leprae into Korean chipmunks.

Exp. Group.	Route of inoculation		No. of total AFB		
			at 2 wks	at 8 months	at 12 months
C	Ear lobe	L**	—*	1.8×10^5	5.7×10^6
			—	8.4×10^5	1.1×10^5
				—	9.5×10^4
				—	—
		R***	—	—	—
			—	—	—
D	Foot pad	L	—	2.9×10^5	1.9×10^5
			—	1.3×10^5	1.5×10^5
				—	5.6×10^4
				—	—
		R	—	—	—
			—	—	—

*:Undetected by the pin head method of Hanks (1968[30]).
**:L (Left); inoculated with unheated Myco. leprae.
***:R (Right); inoculated with heat-killed Myco. leprae.

The residues after the first trypsinization at 37°C for 1 hour was again trypsinized for the second cycle of purification. The first and second cycles of trypsin purification resulted in the yields of 2.4×10^8 of AFB respectively.

2. Growth of trypsin-purified Myco. leprae in the foot pad and ear lobe of Korean chipmunk:

Results of countings of total numbers of AFB in the foot pads and ear lobes of chipmunks, inoculated with trypsin-purified Myco. leprae, at 2 weeks, 8 months and 12 months are shown in Table 2.

In foot pads and ear lobes inoculated with unheated preparation of trypsin-purified Myco leprae, there were apparent increases in total numbers of AFB per tissue at 8 months and 12 months post-inoculation. However, no detectable number of AFB was present in both foot pads and ear lobes inoculated with heat-killed preparation of trypsin-purified Myco. leprae throughout the experimental period. This indicated that a limited but substantial multiplication of trypsin-purified Myco. leprae occurred in those sites.

As previously reported from this laboratory (Lew et al., 1970[24]; Yang and Lew, 1971[25]), the growth of trypsin-purified Myco. leprae in foot pads and ear lobes of Korean chipmunk appeared to be well localized at the sites of inoculation and microscopic examination of stained preparations did not show any apparent pathological changes in foot pads and ear lobes in which multiplication of trypsin-purified Myco. leprae occurred.

DISCUSSION

The aims of trypsin-purification of Myco. leprae from lepromatous nodules in this study are 1) the collection of viable, infectious Myco. leprae, 2) the omission of mechanical damages to Myco. leprae given by grinding in motar and pestle and homogenizing treatment of lepromatous tissues and 3) the removal of contaminating tissue debris from Myco. leprae preparations.

Our purification method resulted in the

collection of sterile purified Myco. leprae from minced tissue of lepromatous nodules.

An unique feature of this procedure is the complete omission of either grinding or homogenization of lepromatous tissues that became a routine in the procedures of purification works by others.

Purified preparation of viable Myco. lepraemurium has been obtained by trypsin treatment of rat leproma tissues (Lew, 1955[8]; Lew and Carpenter, 1956[14]; Nakamura and Ueno, 1963[9]). Ito and Sonoda (1957[17]), in a comparative study on the yields and viability of Myco. lepraemurium obtained by centrifugation, trypsin treatment and centrifugation, and trypsin treatment and Hanks' concentration method(1951[2]), concluded that the method of pretreatment with 0.1% trypsin for 1 hour at 37°C gave the best result and the activity of the bacilli was not affected by this treatment. 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.

Inoculation of trypsin-purified Myco. leprae into either foot pads or ear lobes of Korean chipmunks resulted in multiplication of Myco. leprae. General pattern of multiplication of trypsin-purified Myco. leprae in those sites of chipmunks was very much similar to those of conventionally collected(by grindings in motar and pestle followed by low-speed centrifugation) Myco. leprae in the same animals (Lew et al., 1970[24]; Yang and Lew, 1971[25]).

All of these data including ours strongly indicate that application of trypsin treatment in the procedure of purification of Myco. leprae or Myco. lepraemurium from infected tissues will not exert serious effect on the viability of the organisms.

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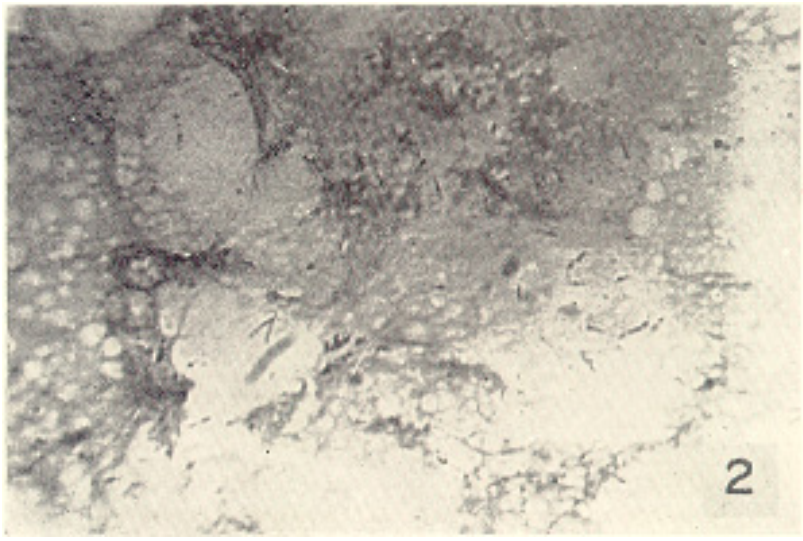

Fig. 2. Bacillary suspension obtained after trypsinization at 37°C for 1 hour of minced lepromatous tissues. A-F stain, 1000×.

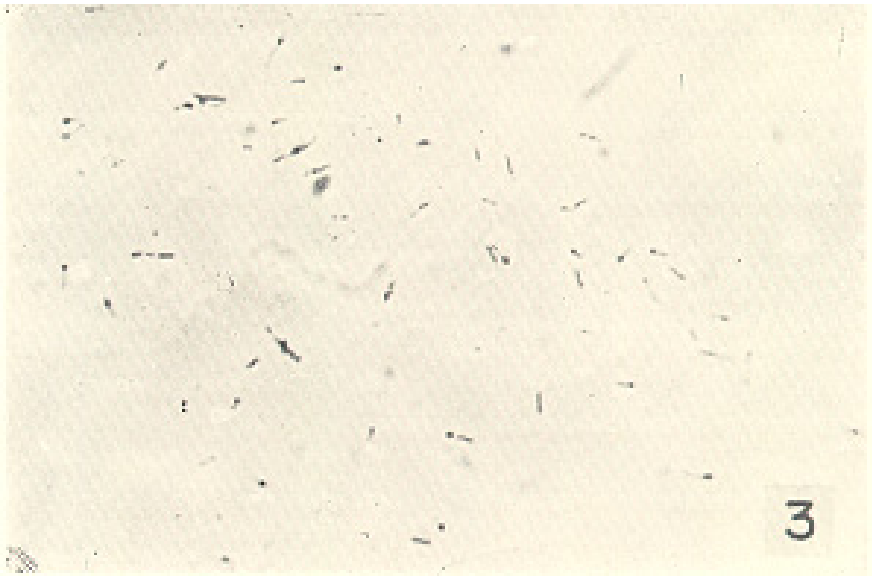

Fig. 3. Final product of trypsin purification of Myco. leprae from the lepromatous tissues. A-F stain, 1000×.