OBSERVATIONS ON THE CULTURE OF *M. LEPRAE* IN MEDIUM V

A Preliminary Report

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This paper reports the preliminary findings of an investigation using Medium V for culture of M. leprae. *The medium was prepared adhering to the procedures adopted by Veeraraghavan. Tissues from 7 cases of lepromatous leprosy (Bacteriological Index of 3 or more) were inoculated into Medium V and incubated at 8–10°C for 72 hours. Quantitative estimation of leprosy bacilli were made employing the enumeration technique of Veeraraghavan. There was no evidence of multiplication in any of the cultures. The findings are in conformity with those of Kato (1983) and of Katoch and Desikan (1983).*

INTRODUCTION

M. leprae, the causative organism of human leprosy, has eluded cultivation *in vitro* for more than a century since its discovery in 1873 by the Norwegian Leprologist Dr. Armauer Hansen. Medical Scientists all over the world have failed in their attempts to cultivate the organism *in vitro.* None of the earlier claims of successful cultivation of *M. leprae* has been confirmed. Failure to cultivate the organism in the laboratory has been a major hurdle to the progress of laboratory studies in leprosy with the result the research has been tardy. Recently, Veeraraghavan (1982) reported successful *in vitro* cultivation of *M. leprae* using a purely synthetic medium, Medium V, formulated by him (1983). The claim has, however, been questioned by Kato (1983) and by Katoch and Desikan (1983). This paper presents our observations on the culture of *M. leprae* using the Medium V.

MATERIALS AND METHODS

Medium V for culture of *M. leprae* was prepared according to the methods described by Veeraraghavan (1983). From each of 7 patients with lepromatous leprosy, skin scrapings from five different active sites were collected by slit and scrape method and were inoculated into the medium. The cultures were incubated at 8–10°C in the lower shelf of a

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domestic refrigerator. Smears were made from cultures as described by Veeraraghavan at 0 hr and at the end of 72 hrs of incubation using 0.01 ml of the culture, accurately pipetted out using a micropipette, with equal volume of formal-milk-serum mixture. Smears were air-dried, heat fixed on the lid of a boiling water bath for 2 minutes and stained by standard cold Ziehl-Neelsen's method.

The bacilli in the smear were enumerated using the method of counting suggested by Veeraraghavan (1983). They were classified in the following manner, namely, single bacillus (SB), small groups (SG 2-10 bacilli), big groups (BG 11-20 bacilli) and very big groups (VBG>20 bacilli). The bacilli in the *entire* smear containing 0.01 ml of the culture were counted. The counts at 0 hr and 72 hrs were compared to compute the degree of multiplication. Bacteriological index and morphological index by Ridley's scale were determined for each patient.

Mouse foot-pad inoculations were carried out using CBA strain of mice to confirm viability of *M. leprae* in the tissues of patients included in the investigation. 5×10^3 acid-fast bacilli in 0.03 ml of Hank's balanced salt solution was used as inoculum per foot-pad. Harvesting of foot-pads will be done at the end of 4 and 8 months of inoculation.

HISTORY OF PREVIOUS TREATMENT IN PATIENTS

Of the 7 lepromatous leprosy patients, one patient (No. 7) had no history of previous chemotherapy for leprosy, one (No. 6) had native treatment for 3 years, 3 patients had specific treatment with DDS for less than 3 months, and 2 patients had DDS for 2 years and 3 years, respectively.

RESULTS

The results of cultures from 7 lepromatous leprosy patients are presented in the Table. According to Veeraraghavan (1983a), a rise in counts of all groups, particularly the single bacillus and groups of 2-10, is evidence of multiplication. None of the 7 cultures showed an increase in all groups. Four showed an insignificant increase in the single bacillus count, namely, 1%, 6%, 20% and 29%, respectively.

The value of Bacteriological Index and Morphological Index are also presented in the Table. It may be observed that all patients had a Bacteriological Index of 3.00 or more and Morphological Index ranged between 0 to 2.08%. The results of mouse foot-pad inoculations are awaited.

TABLE

Results of Cultures from Lepromatous Tissue

	Bact. Index	Morph. Index	Gradings of Cultures			
			VBG	BG	SG	SB
Patient 1:						
0–hr	4.1	0%	277	1037	9226	70639
72–hrs			80	124	1706	2474
Patient 2:						
0–hr	4.5	0.33%	105	159	9241	52798
72–hrs			0	500	4400	8700
Patient 3:						
0–hr	4.3	0%	8	60	944	4962
72–hrs			2	48	720	4408
Patient 4:						
0–hr	4.0	0%	1	9	81	623
72–hrs			0	1	86	804
Patient 5:						
0–hr	3.0	0%	12	64	848	3207
72–hrs			4	34	536	3860
Patient 6:						
0–hr	4.5	2.08%	1	16	2400	17593
72–hrs			3	27	2065	17833
Patient 7:						
0–hr	5.0	0.86%	0	4	135	6951
72–hrs			1	3	98	7371

VBG = Very big groups (21 or more bacilli); BG = Big groups (11-20 bacilli); SG = Small groups (2-10 bacilli); SB = Single bacillus.

DISCUSSION

An organism can be considered as successfully cultivated *in vitro* if there is definite evidence of significant increase in the number of organisms in the culture medium, and the organisms thus grown are confirmed

to be identical with the organism originally inoculated into the medium. The claims made by many workers of having successfully cultivated *M. leprae* could not be substantiated since the organisms grown could not be identified with the original organisms. Skinsnes et al. (1975) reported the cultivation of *M. leprae* from lepromatous leprosy patients but the results were regarded inconclusive without establishing infection in animals. The isolate of Skinsnes (H1-75) was subsequently identified as *M. marinum (M. scrofulaceum)* by Pattyn (1976). Stanford et al. (1977) in London also examined a subculture of this isolate and came to the same conclusion. Kato (1976) had examined the isolate and showed that it was a subspecies of *M. scrofulaceum.* Another group working on *in vitro* cultivation of *M. leprae* had reported spheroidal bodies of leprosy (SPBL) from plasma of lepromatous patients (Barksdale et al. 1973). Attempts at fulfilment of Koch's postulates with SPBL were unsuccessful since negative results were reported for normal mouse foot-pad inoculations and inconclusive results were produced in experiments with Armadillo. Serodiagnosis of *M. leprae* is not sufficiently stringent since it has been shown by Kronvall et al. (1976) that one of the eight antigens associated with *M. leprae* was also present in three slow growing and eight fast growing mycobacterial species and further, an antigen 21A was shared by all mycobacterial strains examined. Thus immunological evidence for *M. leprae* is not sufficiently stringent. Dr. Veeraraghavan (1982) reported successful *in vitro* culture of *M. leprae* in a purely synthetic medium and had stated that there was a 2-6 fold multiplication in 8-60 hrs. Dharmendra (1982), commenting on the findings of Veeraraghavan noted that the multiplication claimed in 18-40 hrs was only 2-6 fold; the claim of rapid growth could not be reconciled with well documented long generation time of *M. leprae,* namely, at least 12 days, as computed from the data from mouse foot-pad experiments. Further, the guidelines prescribed by Veeraraghavan (1983a) for computation of growth in cultures appear to be purely arbitrary and as such could lead to variations between any two readers or even with the same reader with the same smears. Nevertheless, the counts may be considered to be reasonably valid if there has been a uniform increase in large numbers in all the groups and single bacillus at 72 hours. The results of investigation carried out at the Tuberculosis Research Centre, Madras on 7 patients have shown no growth of *M. leprae* in Medium V during 72 hrs of incubation at 8-10°C and the findings are in conformity with those of Kato (1983) and of Katoch and Desikan (1983). Veeraraghavan (1983) has also stated that the Medium V with some modifications gives very good growth of *M. tuberculosis* in 48 and 72 hrs. It is very well known that *M. tuberculosis* can be grown in simple liquid media

such as Tarshis, Youman and Karlson and Kirschner's medium. These media do not require very expensive ingredients as found in Medium V and as such are better for cultivation of *M. tuberculosis* than using a very expensive medium like Medium V.

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