Effects of the Depth of Culture Medium on Elongation of *Mycobacterium lepraemurium In Vitro*[1]

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It is quite well known that elongation of bacteria, in general, is a phenomenon preceding bacterial cell division. *Mycobacterium lepraemurium* is an obligate parasite and cannot be cultivated in cell free medium. Elongation of the bacilli *in vitro* under physiologic conditions may be significant for the studies on cultivation of the bacilli in cell free media. Hart and Valentine (6) first reported the elongation of *M. lepraemurium* in cell free medium under conditions within the limits of physiologic circumstances, and they (6) and Hart (5) and Hilson (8) noted in detail the necessary conditions for elongation of the bacilli.

In a previous paper (10), it was reported that elongation of *M. lepraemurium in vitro*, as observed by the slide culture method, occurred more consistently in the medium at pH 6.0 than at pH 7.0. The slide culture method eliminates two disadvantages involved in the original method (6), namely the centrifugation required for collecting the bacilli and the deformation of the bacilli caused by this centrifugation.

The purpose of this paper is to report further experiments which indicate that elongation of *M. lepraemurium* in cell medium is significantly dependent upon the depth of culture medium in the test tube.

MATERIALS AND METHODS

M. lepraemurium. A bacillary suspension was made from subcutaneous lepromas of a mouse (dd) infected with the Kumamoto strain of *M. lepraemurium*. A homogenate was prepared with sterile saline containing 0.1 per cent bovine albumin V fraction (Armour). A suspension containing an appropriate concentration of bacilli for counting was prepared with the same sterile solution, and was used as the starting material.

Medium used. The basal medium was composed of M/10-M/15 Na_2HPO_4 and KH_2PO_4 mixture (PB) at pH 6, 7, and 8. Usually 0.05 per cent glucose (W/V), 3 per cent glycerine (V/V), 0.08 per cent sodium citrate (W/V) and 0.03 per cent magnesium sulfate (W/V) were added to the basal medium. Thereafter, the medium to be composed of the Sörensen buffer (PB) plus the additives is designated PB-BM. In other words, the PB-BM is the medium in which the casamino acids, asparagine, and sucrose were omitted from the Hart-Valentine medium (6). The media were autoclaved at 120°C for 20 minutes. The culture media thus prepared, with sterile serum at desired concentrations, were distributed into sterile test tubes (10.5 x 1.5 cm.) with rubber stoppers. The net length of the tube from the bottom to the rubber stopper was 9 cm.

Assessment of elongation of bacilli. The general procedure for assessment of *M. lepraemurium* elongation *in vitro* has been described in a previous paper (10). Briefly, each loop of the bacillary suspension was smeared aseptically on a sterile half-size glass slide made by cutting a standard microscope slide lengthwise. The smear was dried at room temperature. After drying, the glass slide was immediately placed in the medium and incubated at 37°C. One smear fixed in 10 per cent aqueous formalin immediately after drying served as an initial reference point. After a specified interval of incubation, the cultivated slides were transferred from the medium to 10 per cent aqueous formalin, washed well with water, and then stained by the Ziehl-Neelsen method.

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TABLE 1. *Relationships between elongation of* Mycobacterium lepraemurium *and storage period of the bacilli after harvest as well as volumes of culture medium.*

Storage period of material at −20°C (days)	Cultivation medium[a]	Volumes of medium per tube (ml.)	Initial length (μ)	Mean length after 18 days' cultivation (μ)	Length increase (fold)[b]
0	PB−BM+10%BS	8	2.2	4.0	1.8
0	PB+20%BS	6	2.2	3.5	1.6
0	"	12	2.2	6.2	2.8
0	PB+10%BS	8	2.2	3.8	1.7
35	PB+20%BS	8	2.1	3.3	1.6
53	PB−BM+10%BS	4	2.0	2.9	1.5
53	"	12	2.0	4.2	2.1
55	PB−BM+20%BS	8	2.1	3.3	1.6
86	PB+10%BS	7	2.3	2.4	1.0
142	PB−MB+10%BS	6	2.0	2.5	1.3
729	PB−BM+10%BS	6	1.8	1.8	1.0

[a] PB = M/15 Sörensen phosphate buffer (pH 6.0), PB−BM = M/15 Sörensen phosphate buffer (pH 6.0) containing additives (see text). BS = bovine serum.

[b] Length increase = $\frac{\text{Mean length after 18 days' incubation}}{\text{Initial length}}$

An appropriate field in the stained smear was photographed on black and white film under the light microscope. The bacillus on the films was enlarged on a print to a final magnification of 1,700. From this the lengths of bacilli could be determined directly by a measuring device.

RESULTS

Relationship between storage period and elongation of bacilli. The period between harvest of bacilli from the mouse leproma until experimental use was investigated for its relationship to the results. The harvested lepromas were usually kept in a deep freezer (−20°C) until used. Additionally, the effects on elongation of varying volumes of media per tube were also tested. Results obtained are presented in Table 1. Elongations were consistently observed in fresh materials shortly after harvest. No elongation occurred in old materials stored for 86 days or more. However, if the bacilli, even though relatively old, were cultivated in large volumes of the medium, elongation occurred. In other words, it is presumed that maintaining the bacilli in a large amount of medium, i.e., under low oxygen tension, is a necessary condition for elongation.

Influence of culture medium volume on elongation of *M. lepraemurium*. Different amounts of culture medium were distributed into the test tubes, in order to see if culture medium depth had any effect on elongation of bacilli. The smear on the glass slide was located between 1.0 cm. and 2.0 cm. from the bottom of the tube. When 2 ml. of medium was poured into the tube, the depth of medium became 1.5 cm., and consequently the air-layer in the tube became 7.5 cm. In the case of medium depths of 4, 6, 8, 10 and 12 ml., the medium to air-layers ratios in the tubes were 3.0:6.0, 4.5:4.5, 6.0:3.0, 7.5:1.5, and 8.5:0.5 cm., respectively.

The results of assessment of elongation under these conditions after 18 days of cultivation in M/15 Sörensen buffer (pH 6.0) with additives (PB-BM) of 20 percent bovine serum medium are shown in Figure 1. The results indicate that the deeper the medium the greater the elongation observed.

These significant effects of volume of culture medium on elongation of the bacilli were further examined as follows. Five and 10 ml. of culture media (PB-BM plus 10% bovine serum) at pH 6, 7, and 8 were used for this purpose. The glass slide bacterial

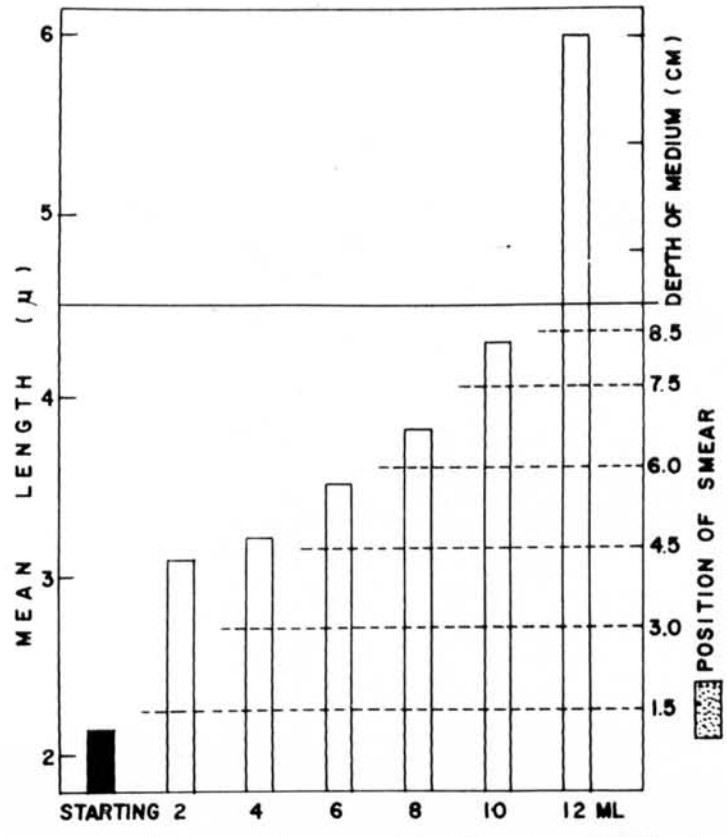

FIG. 1. Relationship between elongation of bacilli and depth of culture medium. Elongation was assayed for 18 days' cultivation at 37°C in M/15 Sörensen buffer (pH 6.0) containing additives and 10 per cent bovine serum. ------ represents the surface of culture medium in the tube. ———— represents the bottom line of a rubber stopper. The net 9 cm. long tubes, as noted in MATERIALS AND METHODS, were used. The bars represent the mean length of bacilli incubated in different volumes of medium illustrated in the figure. The black bar indicates the mean length of the bacilli of starting material.

smears were cultivated for 20, 40 and 60 days at 37°C, and elongations of the bacilli were observed at each cultivating period. The results are presented in Figure 2. The greatest elongation was found in 10 ml. culture medium at pH 6 rather than 5 ml. medium as mentioned above, and even at pH 7 greater elongation was seen in 10 ml. medium volume as compared with 5 ml. of culture medium. No significant elongation was observed at pH 8. Therefore, the conditions essential for elongation of *M. lepraemurium* in cell free medium are freshness of the materials used and cultivation under relatively low oxygen tension in addition to the acidity of the culture medium.

DISCUSSION

Observations of changes in the length of *M. lepraemurium* in the evolution of murine leprosy in mice ([2, 9]), have indicated that elongation of bacilli *in vivo* starts as early as the fourth day after infection and the length of bacilli increases steadily, reaching a maximum of about 2.6 times the initial size on the eleventh day. In tissue culture experiments also, elongation of bacilli has been observed three or four days after infection of mouse peritoneal macrophages ([3, 4]). Therefore, it may be presumed that the phenomenon of elongation of *M. lepraemurium* might be a first step in

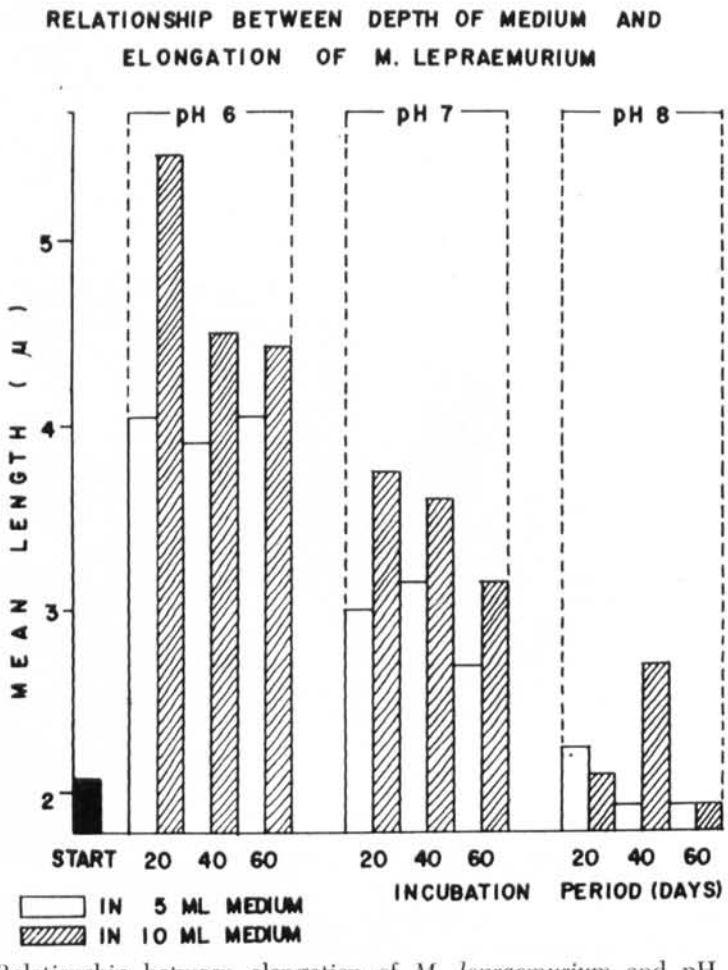

FIG. 2. Relationship between elongation of *M. lepraemurium* and pH as well as volumes of culture medium.

cell division, as can be observed in many cultivable bacilli, although it is known that the length of bacilli increase also when in an unfavorable environment. Hart and Valentine (6) established a specific medium for elongating *M. lepraemurium* in cell-free medium. In this medium, it was established that the mean length of the bacilli doubled in about seven to 14 days and quadrupled in about two months of cultivation at 37°C. Hart (5) indicated that elongation of bacilli is a vital process, rather than a passive stretching or a mere accumulation of material. Evidence for this was provided by the following observations: (1) the bacterial dry weight, as estimated from the electron micrographs, increased in proportion to the length; (2) susceptibility to temperature of cultivation is as might be expected, from bacterial growth; (3) isoniazid suppressed elongation.

The present study gives evidence that the elongation phenomenon was influenced by the depths of culture medium, and implies that the greatest elongation was obtained under relatively low oxygen tensions. This evidence should have a bearing on attempts to cultivate *M. lepraemurium* in cell-free media. The results indicate that conditions optimum for elongation of the bacilli are: the use of fresh murine leprosy bacilli within two months after harvest, and the cultivation of the bacilli under relatively low oxygen tension at pH 6.0 in a medium containing additives mentioned above and 10-20 per cent serum. If these

conditions are maintained, elongation of the Kumamoto strain of *M. lepraemurium* in cell-free medium is observed without exception.

On the basis of the morphologic changes in bacilli *in vivo* observed by Chang [1] and Hart *et al.* [7] it may be presumed that this progressive elongation phase will stop and be followed by shortening, if the bacilli can fission and multiply *in vitro* under appropriate conditions.

SUMMARY

Elongation of *M. lepraemurium* in cell free medium is markedly influenced by the depth of culture medium; the deeper the medium, the greater the elongation observed. If fresh murine leprosy bacilli are used as a starting material and are cultivated in the acid medium established by Hart and Valentine, under relatively low oxygen tension, elongation of *M. lepraemurium* (Kumamoto strain) in cell free medium occurs regularly.

RESUMEN

La elongación del *M. lepraemurium* en un medio libre de células está marcadamente influenciada por la profundidad del medio de cultivo; mientras más profundo es el medio, mayor es la elongación que se observa. Si se utilizan bacilos frescos de lepra murina como material inicial y se cultivan en el medio ácido desarrollado por Hart y Valentine, con una tensión de oxígeno relativamente baja, la elongación del *M. lepraemurium* (cepa Kunamoto) se produce en forma regular en un medio libre de células.

RÉSUMÉ

L'élongation de *M. lepraemurium* dans un milieu sans cellules est influencée de façon notable par la profondeur du milieu de cultures; l'élongation observée est d'autant plus profond. Lorsque des bacilles de la lèpre murine frais sont utilisés comme matériel de départ et cultivés dans le milieu acide établi par Hart et Valentine, sous une tension relativement faible en oxygène, l'élongation de *M. lepraemurium* (souche Kumamoto) en millieu sans cellules survient régulièrement.

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