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Stimulation of Growth of *Mycobacterium lepraemurium* in Cell-Free Liquid Medium by DL-Aspartic Acid[1]

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In order to improve NC (4), NC-5 (5), and ND-5 (6) media, soluble starch (8, 15), and liposome (10) were added to them. The effects of these ingredients on the prolonged growth of *Mycobacterium lepraemurium* were tested because NC, NC-5, and ND-5 media are still useful to obtain primary multiplication of *M. lepraemurium* from an infected mouse. In addition, the effects of amino acids and vitamins (11) on possible continuous growth of *M. lepraemurium* have been tested in the Nakamura system. During these investigations, it was recently found that DL-aspartic acid markedly stimulated the growth of the bacilli in cell-free liquid medium. This interesting result has been reported in preliminary form (12).

The purpose of the present paper is to describe in detail the factors affecting the activity of aspartic acid on the growth of a fastidious microorganism, *M. lepraemurium*.

MATERIALS AND METHODS

M. lepraemurium. *M. lepraemurium* Hawaiian strain (M-71) were obtained from a subcutaneous lepromа experimentally produced in a C_3H mouse. The leproma was homogenized with a glass homogenizer in M/30 Sorensen buffer (pH 7.0) containing 0.1% v/v calf serum. Before use the bacillary suspension was appropriately diluted with Sorensen buffer containing calf serum.

Culture media tested. The compositions of the culture media were as follows:

1) **ND base.**

Dubos powder[3]	0.5 g
adenosine	1.0 mg
thymidine	2.0 mg
thioglycollate	1.0 mg
succinamide	42.0 mg
water	38.0 ml

The pH was adjusted to the values indicated in the tables of the results, then autoclaved.

2) **ND.** To sterilized ND base medium (38 ml), 5 ml calf serum was added aseptically.

3) **ND-5.** The following supplements (3.0 ml) which were previously sterilized by Millipore filtration were added to 40 ml of sterile ND medium. In this case, the ingredients of ND base medium were dissolved in 35 ml of water.

Supplements:

5% α-ketoglutarate	0.5 ml
0.3% l-cysteine HCl	0.5 ml
0.02% hemin	1.0 ml
0.5% cytochrome c	0.5 ml
0.03% NADH	0.5 ml

Hemin was dissolved in 0.01 N NaOH.

4) **NDDLP.** Dextran (MW: 100,000–200,000), 82.0 mg (9) and liposome, 0.6 ml, were added to 38 ml of ND base medium, autoclaved, then 5 ml of sterile calf serum was added (ND containing dextran and liposome).

5) **NDDLP-5.** Dextran, 82 mg, and liposome, 0.6 ml, were added to 35 ml of ND base medium, autoclaved, and then 5 ml sterile calf serum and 3 ml of supplements were added (ND-5 containing dextran and liposome).

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[3] Dubos powder (Eiken Co., Japan) is composed of KH_2PO_4 1.3 g, $Na_2HPO_4 \cdot 12H_2O$ 2.2 g, asparagine 2.0 g, peptone 5.2 g, $MgSO_4$ 0.1 g, ferric ammonium citrate 0.01 g, $ZnSO_4$ 0.0001 g, $CuSO_4$ 0.0001 g, $CaCl_2$ 0.0005 g, malachite green 0.002 g and Tween 80 0.5 g (total 11.3127 g).

TABLE 1. *Effect of DL-aspartic acid on the growth of* Mycobacterium lepraemurium *in the presence or absence of dextran and liposome.*

Base medium	Dextran and liposome	Aspartic acid	pH of medium	Inoculum size $\times 10^6$/tube	Fold increase[a]
ND	−	0.02%	6.0	3.0	14.6
ND	+	0.02%			204.2
ND-5	−	0.02%	6.8	3.1	22.3
ND-5	+	0.02%			188.5

[a] After 6 weeks of incubation at 30°C.

6) **NDLA.** DL-aspartic acid was added to NDDLP medium.

7) **NDLA-5.** DL-aspartic acid was added to NDDLP-5 medium.

Preparation of medium. Dextran, liposome, DL-aspartic acid and dimethyl sulfoxide (DMSO) were added to ND medium before sterilization, the pH was adjusted with 20% NaOH or 1 N HCl, then the medium was autoclaved. Liposomes were prepared as follows: lecithin (Wako Co., Japan), 80 mg and cholesterol, 10 mg (Wako Co., Japan) were dissolved in 2 ml of $CHCl_3$; then the chloroform was evaporated with nitrogen gas. After evaporation, 10 ml of distilled water was added to the lecithin-cholesterol mixture, which was then homogenized by ultrasonication, 19.5 KH_z, 3A (Kaijo Electric Co., Japan, T-A-4280 type) for 5 min. The homogenate was kept in the cold until use.

Menadione (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) was used for preparing vitamin K_3 solution; 8 mg of menadione was dissolved in 10 ml of distilled water, diluted 1000 fold with distilled water, then sterilized by filtration through a Millipore filter (pore size, 0.22 μ). Para-aminobenzoic acid (PABA) (1 mg/ml of H_2O) also was sterilized by filtration.

Cultivation and assessment of the growth of *M. lepraemurium*. Two-tenths of a milliliter of *M. lepraemurium* suspension was inoculated into 43 ml of culture medium, which was distributed in 5 ml or 7 ml quantities into test tubes (10.5 × 1.3 cm) fitted with sterile rubber stoppers. Cultivation was carried out at 30°C. Growth of *M. lepraemurium* was estimated six weeks after inoculation by Hanks' pinhead method ([3]).

Growth rates were calculated by the ratio between the number of bacilli at "0" time and that after six weeks' cultivation. Growth

TABLE 2. *Effect of dose of DL-aspartic acid on the growth of* Mycobacterium lepraemurium.

Base medium	Aspartic acid	pH of medium	Inoculum size $\times 10^6$/tube	Fold increase[a]
NDDLP·K_3	—	6.0	2.3	43.8
	0.01%			216.1
	0.02%			188.0
NDDLP·DMSO-5K_3	—	7.3	8.7	22.9
	0.01%			61.0
	0.02%			64.0
	0.1%			48.9
	0.2%			36.9
NDDLP·DMSO-5PABA	—	6.8	3.5	24.2
	0.02%			175.9

[a] After 6 weeks of incubation at 30°C.

TABLE 3. *Effect of pH on the growth of* Mycobacterium lepraemurium *in culture medium containing DL-aspartic acid.*

Culture medium	pH of medium	Volume of culture medium	Inoculum size $\times 10^6$/tube	Fold increase[a]
NDLA-5	6.0	7 ml	3.2	3.6
	6.3			88.9
	6.6			219.3
	7.0			75.0
	7.3			47.3
NDLA	6.0	7 ml	4.8	100.0
	6.5			58.5
	7.0			13.2
	7.3			3.2
NDLA	6.0	5 ml	4.8	138.2
	6.5			13.1
	7.0			6.6
	7.3			3.1

[a] After 6 weeks of incubation at 30°C.

patterns were morphologically observed by the sediment smear method (SSM) ([7]).

RESULTS

Effect of DL-aspartic acid in the presence or absence of dextran and liposome. DL-aspartic acid was added to ND and ND-5 medium and to the same media containing dextran and liposome, and the effect of aspartic acid on the growth of *M. lepraemurium* was tested. The media were distributed in 7 ml quantities into test tubes. The results obtained are summarized in Table 1. The growth of *M. lepraemurium* was markedly stimulated by the addition of DL-aspartic acid at a final concentration of 0.02% to the media containing dextran and liposome; whereas no effects were observed when aspartic acid was added to plain ND and ND-5 medium. After this experiment, DL-aspartic acid was compared with D- and L-aspartic acid. The results suggest that DL-aspartic acid was slightly more effective than the others, but the differences were not significant.

Effect of concentration of DL-aspartic acid. In order to determine the optimal concentration of aspartic acid, it was added to NDDL-K_3, NDDLP-DMSO-5K_3, and NDDLP-DMSO-5PABA media at final concentrations from 0.01% to 0.2%. The media were distributed in 7 ml quantities into test tubes. The results obtained demonstrate, as shown in Table 2, that 0.01% and 0.02% were optimal. Higher concentrations resulted in a decreased effect.

FIG. 1. Early growth pattern of *M. lepraemurium* grown in NDLA medium, 2 weeks' cultivation at 30°C (high magnification ×1000).

Effect of pH of the culture medium containing DL-aspartic acid. Table 2, row 2 shows that in NDDLP·DMSO-5K_3 medium, aspartic acid had no significant effect. Therefore, it was thought that the effect of pH was much more important for aspartic acid in medium containing dextran and liposome than in plain culture medium. As shown in Table 3, the effect of pH ranging from 6.0 to 7.3 was tested. In the case of NDLA-5 medium, the optimal pH was 6.6. On the other hand, pH 6.0 was the optimum for NDLA medium, with either 7 ml or 5 ml per tube. These results strongly indicate that selection of the optimal pH is the most important factor for the growth of *M. lepraemurium* in media containing dextran, liposome and aspartic acid.

Morphological features of *M. lepraemurium* grown in NDLA and NDLA-5 media.

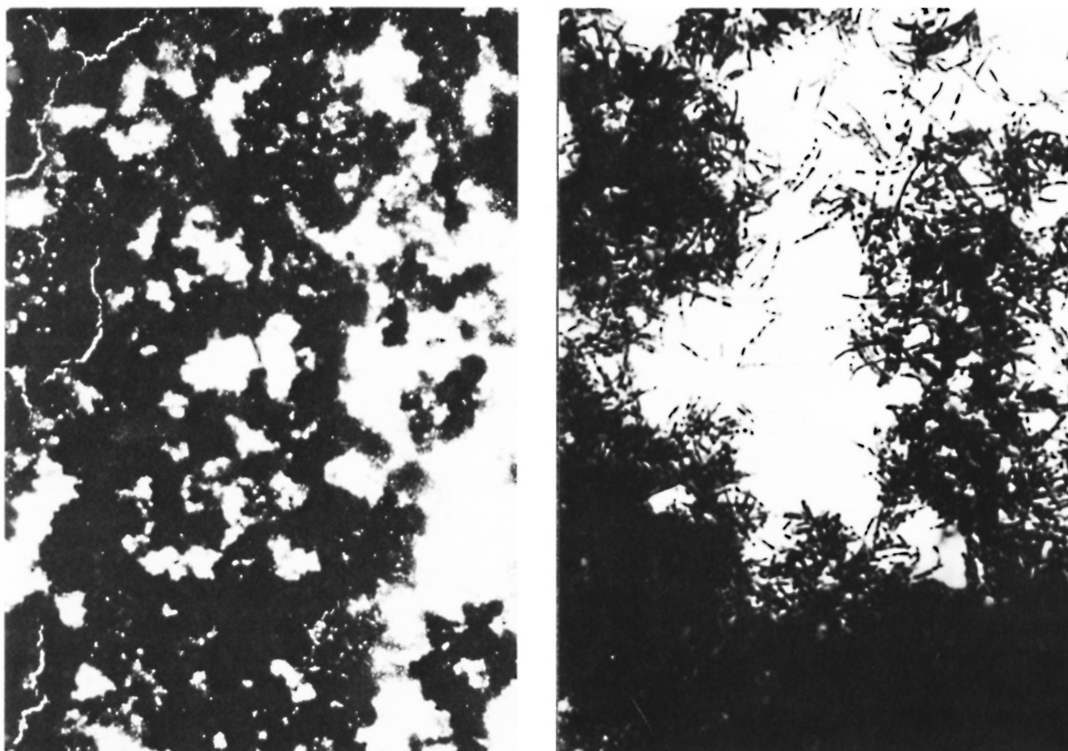

FIG. 2. Morphological features of *M. lepraemurium* grown in NDLA-5 medium, pH 6.6, 7 ml/tube (left: low magnification ×40; right: high magnification ×1000).

The early growth pattern of *M. lepraemurium* cultivated in NDLA medium shows features typical of those usually observed in *M. tuberculosis* (Fig. 1). After six weeks of cultivation at 30°C, however, a majority of the bacilli grown in NDLA at pH 6.0, 7 ml per tube, and in NDLA-5 medium, were nonsolid, beaded, and elongated, and the bacilli were loosely clustered, as shown in Figure 2. On the other hand, bacilli in NDLA, at pH 6.0, 5 ml per tube, were quite solid, and the clumps of bacilli appeared tight (Fig. 3) Therefore, from the point of view of morphological features, it could be considered that the best condition was NDLA medium, pH 6.0, 5 ml per tube.

DISCUSSION

Since 1972 it has been reported that *M. lepraemurium* quantitatively multiplies in the synthetic liquid culture media NC, NC-5, and ND-5 ([4,5,6]). These results were definitely confirmed by Dhople and Hanks ([1,2]). However, it was noted by Dhople and Hanks as well as by the author that growth stopped after six or eight weeks of cultivation at 30°C. Therefore every effort was made to obtain prolonged and continuous growth. Finally Dhople and Hanks (Leprosy Scientific Memoranda 1978, Memo L-938) attained continuous growth of *M. lepraemurium* by substitutions of malic acid, dithiothreitol, and δ-aminolevulinic acid for α-ketoglutarate, cysteine and hemin, respectively. On the other hand, the author had been searching for a growth factor necessary for prolonged growth. During investigations of the effects of amino acids and vitamins, it was found that DL-aspartic acid has a very strong growth stimulating effect ([12]).

As shown in Table 1, the effect of aspartic acid seems to be limited, because the stimulating effect was observed only when aspartic acid was added to medium containing dextran and liposome. It is not yet clear which is essential for aspartic acid, dextran, or liposome, or both.

Besides this problem, there is a most interesting and important problem which

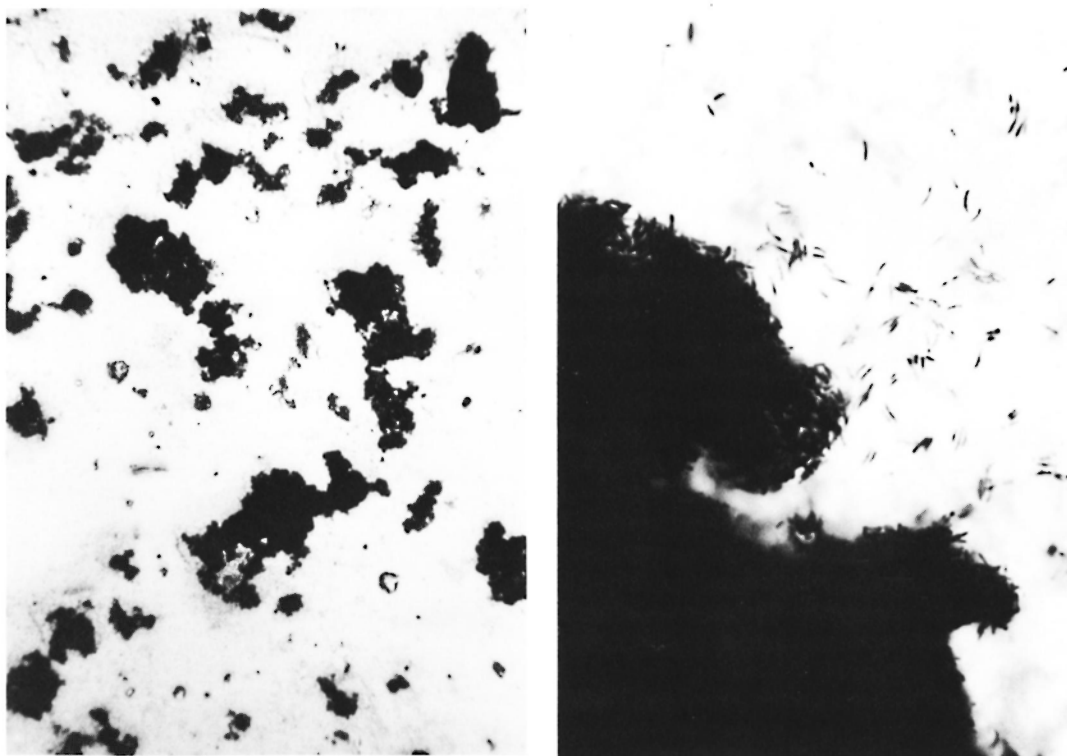

FIG. 3. Morphological features of *M. lepraemurium* grown in NDLA medium, pH 6.0, 5 ml/tube (left: low magnification ×40; right: high magnification ×1000).

should be solved in the future; namely, why asparagine has no such effect, because asparagine is closely related to aspartic acid, and asparagine is frequently used as a nitrogen source for the growth of cultivable mycobacteria. In some cases, asparagine is more effective than aspartic acid. In addition, Dubos powder, which is used in ND base medium, contains asparagine at a final concentration of 0.2% in the complete medium. Nevertheless, no marked stimulating effect like that of aspartic acid was observed in ND medium containing dextran and liposome (Table 2, row 1). However, there is one possible explanation for this fact—that the concentration of asparagine in Dubos medium is too high, 0.2% was slightly toxic even in the case of aspartic acid. In the future, lower concentrations of asparagine should be tested in the NC-5 system ([5]) in which Kirchner medium minus asparagine is employed as the base. According to Kato's report (Leprosy Scientific Memoranda, 1980, Memo L-1117/1), DMSO was tried but it is still not decided whether DMSO is essential or not for the growth of *M. lepraemurium*.

As far as pH is concerned, the author indicated previously that pH 6.8 is optimal in the NC-5 system. In the present study it was also noted that pH 6.6 was optimal in NDLA-5 medium. In contrast, it was strongly indicated that pH 6.0 was optimal in NDLA medium. This value corresponds with those reported by Ogawa and Hiraki ([13]) and Portaels and Pattyn ([14]). The difference in optimal pH between NDLA and NDLA-5 media might be caused by α-ketoglutarate, because the optimal pH of all media containing α-ketoglutarate, for instance, NC-5 and ND-5 medium, is 6.6 to 6.8. This evidence clearly suggests that there might be different metabolic pathways between α-ketoglutarate and aspartic acid. In the future, the effect of aspartic acid should be compared with that of oxaloacetate, because oxaloacetate is much more closely related to aspartic acid than α-ketoglutarate.

Beyond these problems, the data pre-

sented here strongly indicate, as recently pointed out by Portaels and Pattyn ([14]), that the most important and essential factor for the growth of *M. lepraemurium* is optimal pH; the growth is strictly influenced by the pH of liquid medium just as has been demonstrated for solid media, but the optimal pH varies with the composition of the culture medium.

Therefore, three essential factors involved in the Nakamura system, i.e., α-ketoglutarate, 33% air space, and cultivation at 30°C, which were determined by the analytical studies of Dhople and Hanks ([1]), should be changed when a new culture system is established, because, in general, essential factors for cultivation are characterized by different substrates.

Finally, morphological features are known to be important parameters to detect the activity of mycobacteria. The results obtained here demonstrate that the solid cell structure of *M. lepraemurium* was observed when NDLA medium at pH 6.0, 5 ml per tube, was used. An attempt is being made to subculture the bacilli under the conditions described above.

SUMMARY

The growth of *M. lepraemurium* in cell-free liquid medium was strongly stimulated by addition of DL-aspartic acid at a final concentration of 0.01% to 0.02% to ND and ND-5 media containing dextran and liposome. No effect of DL-aspartic acid was observed when it was added to ND and ND-5 media without dextran and liposome. Optimal pH of the culture medium is critical for the stimulating effect of DL-aspartic acid, and it varies with the composition of the medium; the optimal pH was 6.0 in ND medium containing dextran and liposome (NDLA), and was 6.6 in NDLA medium supplemented with α-ketoglutarate, l-cysteine HCl, hemin, and cytochrome c (NDLA-5). A possible mechanism of the effect of aspartic acid is discussed.

RESUMEN

La incorporación de ácido DL-aspártico en una concentración final del 0.01 al 0.02% en los medios ND y ND-5, libres de células, conteniendo dextrana y liposomas, estimuló marcadamente el crecimiento del *M. lepraemurium*. Cuando el ácido DL-aspártico se adicionó a los medios ND y ND-5 en ausencia de dextrana y liposomas, no se observó ningún efecto estimulante. Un pH óptimo del cultivo es crítico para el efecto estimulante del ácido DL-aspártico y varía con la composición del medio. El pH óptimo fue de 6.0 en el medio ND con dextrana y liposomas (NDLA) y de 6.6 en el medio NDLA suplementado con (gamma)-cetoglutarato, l-cisteína HCl, hemina y citocromo C (NDLA-5). Se discute un posible mecanismo del efecto del ácido aspártico.

RÉSUMÉ

La croissance de *M. lepraemurium* dans un milieu liquide sans cellules a été fortement stimulée par l'addition d'acide DL-aspartique à une concentration finale de 0,01 à 0,02%, aux milieux ND et ND-5 contenant du dextran et des liposomes. Il n'a été noté aucun effet de l'acide DL-aspartique à la suite de son addition à des milieux ND et ND-5 sans dextran ni liposomes. Le choix d'un pH optimal pour le milieu de culture est d'une importance critique pour l'effet stimulant de l'acide DL-aspartique; ce pH varie avec la composition du milieu. Le pH optimal était de 6,0 dans le milieu ND contenant du dextran et des liposomes (NDLA), et de 6,6 dans le milieu NDA enrichi en (gamma)-cetoglutarate, de la l-cystéine HCl, de l'hémine, et du cytochrome-c (NDLA-5). On discute du mécanisme possible d'action de l'acide aspartique.

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