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Comparison of Mycobacterial Growth in Dubos Medium, Hyaluronate Supplemented Medium and Umbilical Cord Extract Based Medium*

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This is a report of attempts to compare the growth yields of various species of fastidious mycobacterium including human pathogens and non-pathogens in the conventional Dubos liquid medium and two simple media formulated recently; one is a medium containing 0.1% hyaluronic acid and 6.0% bovine serum albumin and the other is a semisyntheic medium made of umbilical cord extract supplemented with 10% sheep serum as a final concentration.

All mycobacterial strains employed in experiments gave the heaviest growth yields in the hyaluronic acid-bovine serum albumin medium (HAS medium), among the three media. Dubos liquid medium seemed to be inferior to a medium made of umbilical cord extract (UCE medium) in supporting mycobacterial growth. There were three- to seven-fold increases in dry weight of the bacteria grown in the HAS medium as compared with those in the Dubos liquid medium. We also looked for the possible effect of bovine serum albumin (BSA) in the HAS medium on mycobacterial growth. As a result, we found that the amount of BSA in the HAS medium, ranging from zero to 6.0% in the medium, showed no substantial effect on the mycobacterial growth.

There have been a great number of attempts by leprologists to cultivate the causative agent of human leprosy, *M. leprae in vitro* since its discovery by Hansen in 1874, but none of them have been very successful. At present, we do not have dependable experimental animals, although there are few reports on animal models for *in vivo* multiplication of *M. leprae* (Shepard, 1960, 1962, Kirchheimer and Storrs, 1972, Lew *et al*, 1974).

A few years ago, Skinsnes and his associates (Skinsnes *et al*, 1974) performed histochemical studies of leprosy skin biopsies and reported that there was an abundant deposit of hyaluronic acid (acid mucopolysaccharide with altering beta 1-3 glucuronidic and glucosaminidic bonds) in human lepromas. On the basis of this observation, they made a suggestion that *M. leprae* may utilize hyalu-

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ronic acid as a nutrient for its growth in leproma cells.

Subsequently, they looked for possible effects of hyaluronic acid on the *in vivo* growth of murine leprosy bacillus, *Mycobacterium lepraemurium*, which is considered similar to the human leprosy bacillus in some aspects (Matsuo *et al*, 1975). From this study, they claimed that hyaluronic acid seemed to enhance *in vivo* multiplication of *M. lepraemurium*. In the same year, the same group reported that they were successful in cultivating *M. leprae in vitro* on a semi-synthetic medium supplemented with 0.1% hyaluronic acid and 6.0% bovine serum albumin (Skinsnes *et al*, 1975), but their result has not been verified yet by others.

Since there has been no good luck in finding suitable experimental animals or in *in vitro* cultivation of human leprosy bacilli, studies on the physiology, infectivity and other properties of this organism are still in their infancy. Regardless of the validity of the sucess of *in vitro* cultivation of *M. leprae* on hyaluronic acid supplemented medium by Skinsnes' group, we became interested in the possible role(s) of hyaluronic acid as a nutrient for *M. leprae*. Also we have thought that hyaluronic acid may serve as a nutrient to other groups of nutritionally fastidious mycobacteria. If so, understanding the metabolism of hyaluronic acid by fastidious mycobacteria may give us a short cut to the study of some properties of *M. leprae*. As the first step toward understanding the metabolism of hyaluronic acid by fastidious mycobacteria, we attempted to grow various species of mycobacteria in hyaluronic acid based simple medium and compared the growth yield with that of the mycobacteria grown in conventional Dubos medium. Since human umbilical cords are rich in hyaluronic acid and the cost of commercially prepared hyaluronic acid is high, a medium made of extracts of umbilical cords was also examined in cultivating fastidious mycobacteria. We looked for an effect of bovine serum albumin contained in the HAS medium on mycobacterial growth.

MATERIALS AND METHODS

Mycobacterial strains: The species of mycobacterium employed in the experiments were as follows; *Mycobacterium fortuitum* 6841, *M. intracellulare* 13950, *M. kansasii* 765, *M. marinum* 977, *M. phlei* 19249, *M. scrofulaceum* 19981 and *M. tuberculosis* $H_{37}Rv$. All strains were orginally derived from those harbored in the JACC (Japaness Association of Culture Collection) or at the Dept. of Bacteriology, Hiroshima University, School of Medicine, Hiroshima, Japan and maintained on Ogawa slants for years at the authors' department.

Media: Dubcs liquid medium was prepared as directed in the Difco manual (9 th edition, Difco Laboratories, Detroit, Michigan, USA). A medium containing 0.1% hyaluronic acid (Sodium salt, Grade Ⅲ-S, Sigma Chemical Company, St. Louis, Mo., USA) and 6.0% (w/v) bovine serum albumin (Cohn fraction V, Sigma Chemical Company) in 66 mM of Na_2HPO_4-KH_2PO_4 buffer, pH=6.24, was prepared in the way suggested by Skinsnes *et al* (1975) with a slight modification, that is, omitting potassium penicillin G and adding 0.6% (w/v) yeast extract powder (Difco laboratories) as a final concentration in the medium. Extracts from umbilical cords were made following digestion of small pieces of the frozen cords with 1.0% (w/v) of 1 : 250 trypsin (Difco laboratories) in 66 mM of Na_2HPO_4-KH_2PO_4

buffer, pH=7.5 for 2 hours at 37°C and filtration through nylon gause. After adjusting the filtrate to pH=6.2 with 2 N HCl, the preparation was autoclaved for 15 minutes at 15 lbs pressure and immediately filtered through Whatmann 3 mm filter paper to clarify extracts. Then thirty grams of glycerin and six grams of yeast extract powder were added to batches of umbilical cord extracts and the final volume was brought to one liter with 66 mM phosphate buffer, pH=6.24. This preparation was rendered as a medium base and sterilized in an autoclave for 15 minutes at 15 lbs pressure. Sterile sheep serum was aseptically added to the medium base to give 10% (v/v) of a final concentration. This medium was designated as UCE medium. Quantitation of hyaluronic acid in umbilical cord extracts was spectrophotometrically made by an enzymatic method (Greiling, 1965) in presence of hyaluronidase (Type Ⅱ, Sigma Chemical Company).

Cultivation of bacteria: Colonies of mycobacterial strains grown on Ogawa slants were transplanted into 3-ml of Dubos liquid medium and cultivated for a week. Three-tenth ml of week old cultures of each mycobacterial strains was inoculated into 5-ml of the three different media mentioned above in duplicate and incubated at the optimal growth temperature for each species. Culture tubes were occassionally shaken by hand for aeration.

Estimation of bacterial growth yields: Bacterial cells cultivated for the specified incubation time collected by centrifugation at 2,000 rpm for 30 minutes (International Portable Refrigerated Centrifuge, Model PR-2, International Equipment Comp., Needham Heights, Mass., USA) and washed twice with each 10-ml of 66 mM Na_2HPO_4-KH_2PO_4 buffer, pH =7.5. The washed cell pellet was resuspended in 2-ml of the same buffer and then dried in a 70°C dry oven for several hours. The growth yield of each mycobacterium in the three different media was compared on the basis of the dry weight in milligrams of the cells.

RESULTS

Growth yields of mycobacteria: Table 1 shows dry weight of various species of mycobacteria grown in different media. As shown in Table 1, all species of mycobacterium gave higher growth yields in the hyaluronic acid-bovine serum albumin containing simple medium (HAS medium) as compared with growth in either Dubos medium or the medium made of umbilical cord extracts (UCE medium). Cell yields of mycobacteria in the HAS medium varied between species. The growth yields of mycobacteria in the UCE medium seemed to be influenced by the amount of hyaluronic acid

Table 1. Compaison of Mycobacterial Growth in Different Media

Strain	Cultivation Period	Media		
		Dubos¢	HAS*	UCE#
M. fortuitum	4 W	25.3@	121.9	63.7
	6 W	20.2	149.5	77.9
M. intracellulare	4 W	23.7	114.1	90.1
	6 W	20.2	119.6	111.6
M. kansasii	4 W	37.5	131.8	62.9
	6 W	33.4	158.7	77.6
M. marinum	4 W	23.5	52.6	40.1
	6 W	21.9	64.3	54.1
M. phlei	4 W	44.5	168.6	147.6
	6 W	31.1	119.5	80.4
M. scrofulaceum	4 W	30.5	105.3	121.3
	6 W	23.4	93.3	133.9
M. tuberculosis H_{37} Rv	4 W	28.8	82.9	53.9
	6 W	28.6	74.5	74.2

¢ : Dubos Liquid Medium
* : Hyaluronic Acid Supplemented Medium,
# : Umbilical Cord Extract Medium
@ : Dry Weight in Milligram (mg)

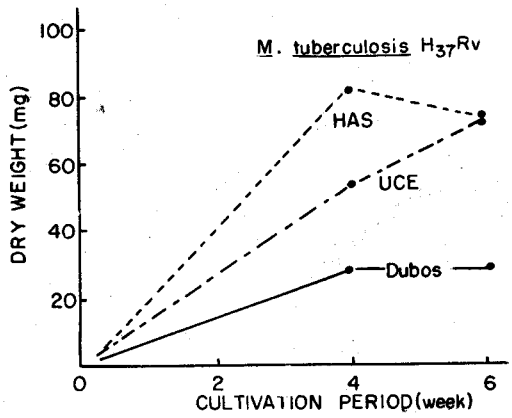

Fig. 1. Growth of *M. tuberculosis* H_{37} Rv in Dubos, HAS and UCE Media.

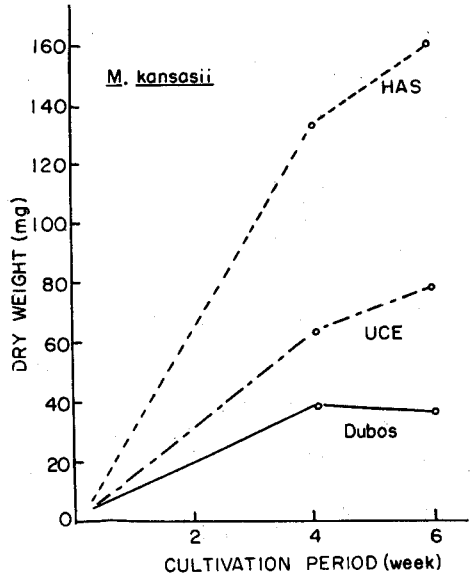

Fig. 2. Growth of *M. kansasii* in Dubos, HAS and UCE Media.

in the batches of extracts. The amount of hyaluronic acid in the batches chosen for the medium used to obtain the data of Table 1 was approximately 700~800 μg per ml, whereas that of the HAS medium was 1,000 μg per ml. Dubos liquid medium, which has been widely used in the cultivation of mycobacteria, appeared to be inferior even to the UCE medium in supporting mycobacterial growth as shown in Figures 1 and 2. These figures were plotted with the data shown in Table 1.

Effect of bovine serum albumin in the HAS medium on mycobacterial growth. In order to see the effect on the growth of mycobacteria of bovine serum albumin added to the hyaluronic acid based simple medium, we varied the amount of bovine serum albumin in the HAS medium from zero to 6.0%. Strains of *M. fortuitum*, *M. intracellulare*, and *M. phlei* were cultivated in these media. Growth of these bacteria was measured in dry weight after one and two weeks cultivation at 37°C. Table 2 shows the growth yields of these organisms grown in the hyaluronic acid based media with different concentrations of bovine serum albumin. As shown in Table 2, growth of the mycobacteria did not seem to correspond to the concentration of bovine serum albumin in the medium, since bacterial growth in the medium without BSA at all is about the same as that with 6.0% BSA (Fig. 3). This became even more apparent when the data of Table 2 were plotted in figure form (Fig. 4). Each spot of cell yields obtained in different concentrations of BSA, 0.0, 0.6, 1.0, and 3.0% (w/v) did not deviate much from

Table 2. Mycobacterial Growth in Hyaluronic Acid Based Media Containing Various Amount of Bovine Serum Albumin

Strain	Cultivation Period	BSA Conc. (%) 0.0	0.6	1.0	3.0	6.0
M. fortuitum	1 W	29.3*	34.4	32.4	29.7	34.4
	2 W	43.3	41.9	43.2	44.8	44.9
M. intracellulare	1 W	37.7	34.7	30.9	36.5	39.3
	2 W	51.4	53.6	43.7	40.8	46.9
M. phlei	1 W	31.8	27.5	28.5	ND	31.7
	2 W	57.0	49.9	59.9	52.8	49.5

*: Dry Weight in Milligram (mg)
ND : Not Determined

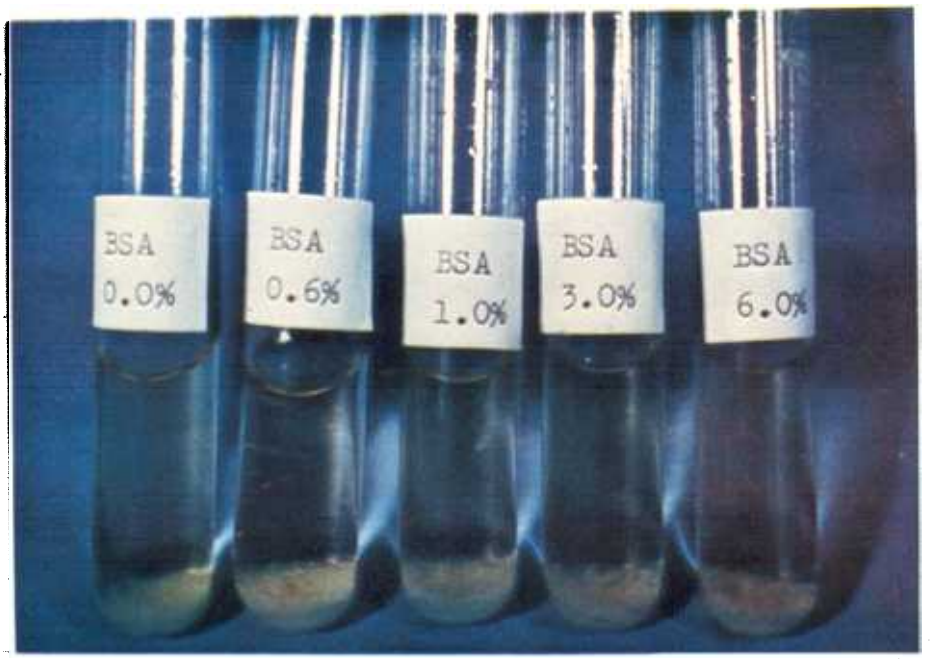

Fig. 3. Growth of *M. intracellulare* in Hyaluronic Acid Based Medium with Different Concentration of Bovine Serum Albumin.

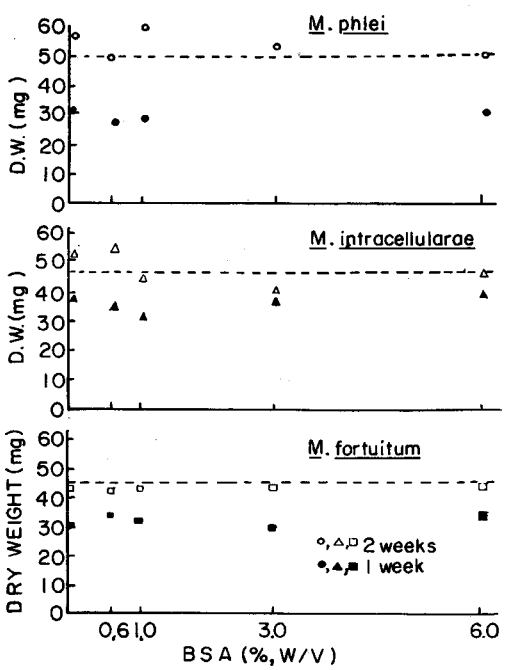

Fig. 4. Bacterial Growth in Different Conc. of BSA in Hyaluronic Acid Based Medium.

the spot in 6.0% BSA in the hyaluronic acid supplemented medium.

DISCUSSION

A number of new species of mycobacterium causing diseases in human subjects have been reported during the last few decades (Barksdale and Kim, 1977), but most of them are poorly understood with respect to infectivity, physiology, antigenic structure and other properties. This is probably due to the fact that the principle mycobacterial pathogens are intracellular parasites. One would speculate that certain components of cells or tissue, for instance, hyaluronic acid, may be related to *in vivo* multiplication of mycobacteria. Projection of such a speculation is also possible on the basis of the observation that cutaneous syphilomas filled with tremendous numbers of *Treponema pallidum*, which multiplies in tissue, contain copious amounts of hyaluronic acid.

Attention to the growth of fastidious myco-

bacteria in relation to hyaluronic acid has been gradually increased (Kato and Ishaque, 1976), after the report on deposits of hyaluronic acid in human lepromas by Skinsnes *et al* (1974).

Our study on the growth of various species of mycobacterium in a medium based on hyaluronic acid suggests to us that hyaluronic acid may serve as a nutrient, most likely a carbon source to mycobacterial growth. Since the medium containing hyaluronic acid, regardless of the bovine serum albumin in it, seemed to be better at supporting mycobacterial growth compared with Dubos medium, this medium could be useful in early bacteriological confirmation of the diagnosis of mycobacteriosis. One may readily replace the hyaluronic acid based medium with the UCE medium for this purpose at low cost.

At present, we do not know the exact role (s) of hyaluronic acid in the growth of mycobacteria. Investigations of the role (s) of hyaluronic acid in the growth of mycobacteria and the metabolic pathways of this substrate by the organisms are worthy of pursuit in order to understand the fastidious nature of mycobacteria, especially *M. leprae* in cultivation.

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