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Studies on the Mycobacteria Isolated from Soil

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Acid-fast microorganisms were isolated from 240 soil samples collected at two areas, Hiroshima, Japan and Seoul, Korea. The biological and biochemical characteristics of the isolated mycobacteria were tested and compared with those of 36 reference mycobacteria strains. The isolation rate and distribution of these mycobacterial species from soil were compared using three kinds of media with emphasis on the two methods of isolation between the different geographical areas. One strain from each of the 10 species among atypical mycobacteria isolated from soil in both areas was inoculated into ddY mice and the pathogenicity compared with that of *Mycobacterium tuberculosis* $H_{37}Rv$ up to 6 weeks.

Susceptibility of the reisolated acid-fast bacilli to antimycobacterial agents was tested *in vitro*. Antibody responses against various mycobacterial antigens were tested using lepromatous type and tuberculoid type patient sera by the agar gel immunodiffusion. 1) No significant differences in the distribution of acid-fast bacilli were observed between soil samples from the two regions. 2) Rapid growers were by far the most frequent acid-fast bacilli isolated while no photochromogens were isolated from these soil samples. In addition, a minimal number of fastidious mycobacteria were isolated but not cultivable in subcultures. 3) Some of these soil acid-fast bacilli were capable of inducing only transient bacteriological and pathologic changes in mouse organs. 4) Acid-fast bacilli reisolated from organs of these infected mice were, in general, found to be resistant to antimycobacterial agents. 5) *M. scrofulaceum* antigen showed a precipitation reaction in agar gel immunodiffusion with the highest number of sera from leprosy patients.

An acid-fast microogranisms, which have different characteristics from those of *Mycobacterim tuberculosis* or *Mycobacterium leprae,* were isolated by Pinner(1935) from a tuberculosis patient showing different clinical manifestations. These microorganisms were called atypcial acid-fast microorganisms and they were pathogenic like the usual pathogenic acid-fast microorganisms.

Urabe(1944) indicated that some acid-fast bacilli(AFB) which had been considered as non-pathogenic contaminants were pathogenic, to some degree, in humans. No extensive search for the pathogenic AFB had been made until pulmonary disease due to AFB in humans were reported by Pollak and Buhler(1951), Buhler

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and Pollak(1953), Timpe and Runyon(1954), and Urabe and Saito(1966).

Presently, many effective anti-tuberculosis and anti-leprosy drugs have been developed. However, mycobacteriosis due to AFB other than tuberculosis and leprosy are being increasingly reported(Brock *et al.*, 1960, Kubica *et al.*, 1963; Bjerkedal, 1967; Adams, *et al.*, 1970; Abello, *et al.*, 1971; Champman, 1971; 1971). Because several AFB inhabit our environment and because a number of researchers have isolated atypical mycobacteria(AM) from soil, we are temped to make an assumption that *M. tuberculosis*, *M. leprae* and other AM came from soil microorganisms evolutionally. Although these AM have been isolated, not one solid hypothesis as to where these AM originated and their modes of infection and pathogenesis in human have been elucidated.

The present study was undertaken to observe the relationship between AM from soil and those from the human. Firstly, methods for the isolation of AFB from soil and types of culture media used were reviewed. Secondly, characterization of isolated AFB and the experimental infectivity of these AFB to mice were examined. Thirdly, the sensitivity of these AFB to anti-tuberculosis agents was determined, and fourthly, immunodiffusion assay was employed to demonstrate antibodies reacting with soluble antigens prepared from isolated AM.

MATERIALS AND METHODS

Soil ; Soil samples from 240 different sites, 120 from Yonsei University campus in Seoul, Korea and 120 from Hiroshima University campus in Japan, were collected.

Medium and Reagent ; Söhngen medium (Söhngen, 1913) and trypticase soy broth (Wolinsky and Rynerson, 1968) were used for the concentration of AFB, while 1% or 3% Ogawa egg medium, Löwenstein-Jensen medium (L-J), and Amiercan Trudean Society medium (ATS) were used for the isolation of AFB. Dubos liquid medium or Sauton liquid medium were employed as a primary medium for the study of biochemical characteristics of these isolated AFB, and all reagents used were "reagent grade". *In vitro* sensitivity tests were performed with anti-tuberculosis agents including streptomycin(SM; Meiji Co., Lot. No. SS-478), isonicotinic acid hydrazide(INH; Takeda Co., Lot. No. 148-ONK), para-aminosalicylic acid (PAS; Tanabe Co., Lot. No.2492 PM52), ethionamide(TH; Daiichi Co., Lot., No. 3N0481), cycloserine(CS; Daiichi Co., Lot. No. C-592), ethambutol(EB; Leaderle Co., Lot. No. 9673-496-1), and rifampicin(Daiichi Co., Lot. No. 9041).

Strains ; *M. tuberculosis* $H_{37}Rv$ and 35 mycobacteria strains which had been maintained at the Department of Bacteriology, Hiroshima University School of Medicine were used as a standard strain and bacterial mass or suspension prepared with AFB, cultured in 1% Ogawa egg medium, was used as a comparative strain. *M. scrofulaceum*, *M. gordonae*, *M. agri*, *M. smegmatis*, *M. aurum*, *M. fortuitum*, *M. terrae*, *M. chelonei subsp. chelonei*, *M. thermoresistibile* and *M. nonchromogenium* which were isolated in Seoul and Hiroshima were selected as experimental strains and for the comparative strain, *M. tuberculosis* $H_{37}Rv$ strain was employed.

Experimental animals ; Five week-old ddY male mice, weighing 25±2 *gm*, had been observed for a week before being used. Healthy mice(12 experimental groups, 12 mice per group) were selected and fed on commerical pellets.

Antigen preparation and serum ; *M. agri*, *M. aurum*, *M. gordonae*, *M. scrofulaceum*, and *M. nonchromogenicum* isolated fro soil were cultured in Sauton liquid media for two to six weeks. And then, filtrates from this culture were

concentrated and sued as antigens. Twenty-nine leprosy patients(22 lepromatous and 7 tuberculoid types) from World Vision Special Skin Clinic, Seoul, Korea were used as serum donors. These sera were kept frozen at -25°C until used.

Isolation of AFB ; Soils, treated according to the Wolinsky and Ryneason method and Sõhngen method(Figure 1 and 2), were inoculated on culture media. The first appearance and morphology of colonies were made up to 8 weeks; daily for the first week and therafter once a week. After confirming that the formed clony was due to AFB with acid-fast stain (A-F stain), the colony was treated with 2% sodium hydroxide solution and 0.1 *ml* of this AFB suspension was reinoculated on the same medium for isolation of AFB.

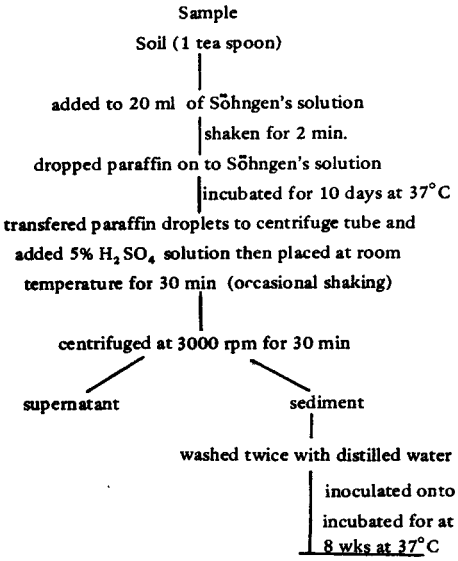

Fig. 1. Isolation method by Sõhngen's (1913).

Runyon groups ; Pure cultures were grown under five different conditions; at 37°C in a dark room, at 28°C, at 33°C, at 45°C, and at 52°C. For 6 weeks, the colonies were observed for the time of formation, morphology, and pigmentation of colonies in order to be classified according to Runyon's grouping(Runyon, 1959).

Biochemical characteristics ; After getting

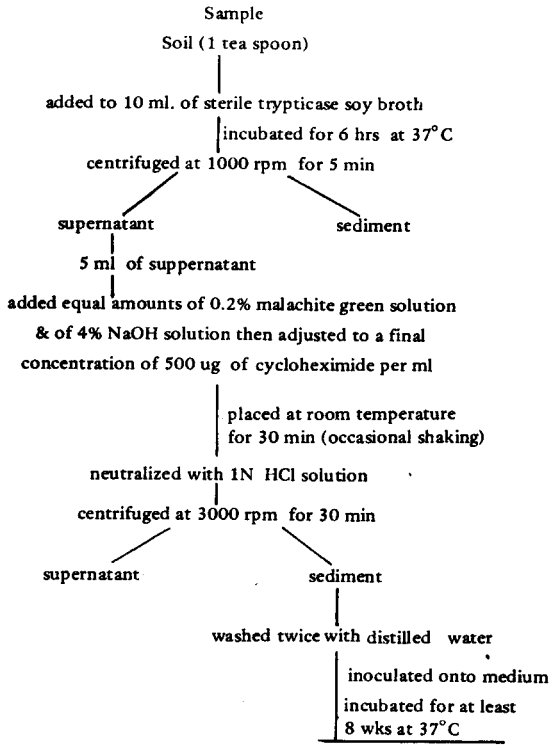

Fig. 2. Isolation method by Wolinsky's (1968).

pure cultures of AFB, niacin test (Kono *et al.*, 1958; Runyon *et al*, 1959) arylsulfatase test (Kubica and Vestal, 1961 Tarshis, 1963), Tween 80 hydrolysis test(Wayne *et al.*, 1964), acid-phosphatase test(Saito *et* al., *1968),* catalase test(Kubica *et al.*, 1966), nitrate reduction test (Virtanen, 1960; Tsukamura, 1967; 1969), neutral red test(Dubos and Middlebrook, 1948; Maeda, 1965), carbohydrate and mineral acid utilization test, amidase utilization test(Tsukamura, 1975) and *in vitro* sensitivity tests to anti-mycobacterial agents were performed. Cultured AFB were classified according to the results from these biochemical tests and compared with those of standard strains (Saito, 1975).

Animal inoculation ; AFB colonies, grown on 1% Ogawa egg medium for 1 to 3 weeks, were taken with the help of a platinum loop and 1 mg of AFB was suspended in sterile distilled water containing 0.1% Tween 80. Then, 0.2

ml of this suspension was injected intravenously into the tails of mice. The number of mice injected with AFB is shown in table 5.

While changes in body weight of injected mice were being observed, 2 mice a week up to the 6th week were sacrificed to evaluate gross changes in the organs of these mice. Root spleen index with the change of spleen weight and root lung index with the change of lung weight were calculated according to the methods of Kudo *et al.* (1970) and Sato *et al.* (1967). AFB isolated from infected organs were re-cultured and *in vitro* sensitivity tests to anti-mycobacterial agents were performed with these re-cultured AFB.

To observe the histopathological changes of infected organs from sacrificed mice at the 4th and 6th week, tissues were fixed in 10% buffered formalin. After hematoxylin-eosin(H-E) staining and A-F staining, pathological changes and the presence of AFB were observed according to methods of Chang *et al.* (1971) and Choi *et al.* (1972).

Agar-gel immunodiffusion ; Agar plates were made with 0.8% agar in phosphate buffered saline(pH 7.2) using pertri dishes of 100 mm diameter following the method of Campbell *et al.* (1972). The precipitation reaction was observed after incubation for 4 to 5 days at 37°C.

RESULTS

Isolation of AFB from soil ; A number of isolation methods and culture media were employed to isolate AFB from 240 different soil samples, 120 samples from Hiroshima, Japan and 120 samples from Seoul, Korea.

Table 1. Isolation of acid-fast bacilli(AFB) with 120 soil samples each from Hiroshima and Seoul

Methods		Sõhngen				Wolinsky			
Media		Ogawa		L-J	ATS	Ogawa		L-J	ATS
Weeks	Area	1%	3%			1%	3%		
<1	Hi*	45**	40	33	31	44	33	29	36
	Se*	37	29	26	21	39	27	14	24
1 – 2	Hi	9	7	8	2	7	6	6	20
	Se	14	9	13	8	11	4	2	6
2 – 3	Hi	2	1	0	0	0	1	1	1
	Se	4	2	4	4	9	3	1	1
3 – 4	Hi	0	0	0	0	2	1	0	0
	Se	0	0	0	0	10	4	0	1
4<	Hi	0	0	0	0	0	0	0	1
	Se	0	0	0	0	1	2	1	1
Total (%)	Hi	56 (46.7)	48 (40.0)	41 (34.2)	33 (27.5)	53 (44.2)	41 (34.2)	36 (30.0)	39 (32.5)
	Se	55 (45.8)	40 (33.3)	43 (42.9)	33 (27.5)	70 (58.3)	40 (33.3)	18 (15.0)	33 (27.5)

*Hi : Hiroshima in Japan, Se : Seoul in Korea

** : Number of positive culture

Among the four culture media used, 1% Ogawa egg medium was the one which gave the highest percentage of AFB isolation. Seventy(58.3%) out fo 120 soil samples from Seoul, Korea gave positive cultures for AFB when the Wolinsky and Ryneason method was employed(Table 1).

No significant differences could be observed on the percentage of positive cultures among soil samples from Hiroshima, Japan and from Seoul, Korea. However, more rapid growers among samples from Hiroshima, Japan and more slow growers among samples from Seoul, Korea were isolated. Söhngen method turned out to be a relatively good method for the initial isolation step with soil samples from both countries, but it was not an adequate medium when more than a 3-week observation period was necessary. Nevertheless, detection of colonies after a 4-week incubation period was possible when the Wolinksy and Ryneason method was employed. Also, it was not possible to eliminate contaminants when the Söhngen method was used. Less contaminants were observed when the Wolinsky and Ryneason method was employed and it was possible to observe colonies even after 3 weeks inoculation.

Runyon grouping of isolated AFB ; Pure culture strains isolated on the Söhngen method were classified according to the Runyon grouping of AFB(Table 2). Among 178 strains isolated from soils from Hiroshima, Japan; 147 strains (82.6%) of rapid growers, 17 strains(9.6%) of scotochromogens, and 8 strains (4.5%) of nonphotochromogens were observed. Among 171 strains isolated from soil samples from Seoul, Korea, 129 strains (75.4%) of rapid growers, 21 strains (12.3%) of scotochromogens, and 14 strains (8.2%) of nonphotochromogens were isolated. Interestingly, not a single strain of photochromogen was isolated among samples of both regions. All isolated AFB were subcultivable except 7 strains in 1% Ogawa egg media, 2 strains in L-J media, and 4 strains in ATS media. The effect of temperature on *in vitro* growth of AFB, when different media

Table 2. Runyon's grouping with the acid-fast bacilli subcultured

Area	Hiroshima					Seoul				
Media / Runyon's Group	Ogawa 1%	Ogawa 3%	L-J	ATS	Total (%)	Ogawa 1%	Ogawa 3%	L-J	ATS	Total (%)
No. of strains	56 (100)	48 (100)	41 (100)	33 (100)	178 (100)	55 (100)	40 (100)	43 (100)	33 (100)	171 (100)
Group – I	0	0	0	0	0	0	0	0	0	0
Photochromogen	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)
Group – II	5	5	5	2	17	7	5	2	7	21
Scotochromogen	(9.6)	(10.4)	(12.2)	(6.1)	(9.6)	(12.7)	(12.5)	(4.7)	(21.2)	(12.3)
Group – III	4	3	1	0	8	5	4	4	1	14
Nonphotochromogen	(7.1)	(6.3)	(2.4)	(0.0)	(4.5)	(9.1)	(10.0)	(9.3)	(3.0)	(8.2)
Group – IV	44	40	33	30	147	39	31	37	22	129
Rapid grower	(78.5)	(83.3)	(80.5)	(90.9)	(82.6)	(70.9)	(77.5)	(86.0)	(66.7)	(75.4)
No growth in subculture	3	0	2	1	6	4	0	0	3	7
	(5.4)	(0.0)	(4.9)	(33.0)	(3.3)	(7.3)	(0.0)	(0.0)	(9.1)	(4.1)

Table 3. Growth pattern of subcultured acid-fast bacilli (AFB) at different temperatures

Media		No. of subcultured strain		Temperatures (°C)									
				28		33		37		45		52	
		Hi	Se	Hi	Se	Hi	Se	Hi	Se	Hi	Se	Hi	Se
1 % Ogawa	Sl :	9	12	7	10	9	12	9	12	0	0	0	0
	Ra	44	39	44	39	44	39	44	39	17	10	3	2
3 % Ogawa	Sl :	8	9	7	9	8	9	8	9	0	0	0	0
	Ra :	40	31	40	31	40	31	40	31	19	6	5	2
L – J	Sl :	6	6	5	4	6	6	6	6	0	0	0	0
	Ra :	33	37	33	37	33	37	33	37	17	8	4	3
A T S	Sl :	2	8	2	7	2	8	2	8	0	0	0	0
	Ra :	30	22	30	22	30	22	30	22	15	6	3	1
Total	Sl :	25	35	21	30	25	35	25	35	0	0	0	0
	Ra :	147	129	147	129	147	129	147	129	68	30	15	8

Sl : Slow grower of AFB, Ra : Rapid grower of AFB, Hi : Hiroshima, Japan, Se : Seoul, Korea

were used, is shown in Table 3. None of the 25 slow growers from Hiroshima soil samples and none of the 35 strains from Seoul soil samples grew at 45°C or higher. However, 68 strains at 45°C and 15 strains at 52°C were cultivated from the 147 strains of rapid growers from Hiroshima soil samples; while 30 strains at 45°C and 8 strains at 52°C were cultivated from the 129 strains of rapid growers from Seoul soil samples.

Characterization of pure-cultured AFB ; All biochemical characteristics of pure-cultured AFB from both regions and standard strains are shown in Table 4. Among AFB strains from both regions, the most frequent cultivable strain was *M. fortuitum* (40.0%) and the second most frequent one was *M. scrofulaceum*, but *M. tuberculosis*, M. *bovis*, and *M. intracellulare* were not observed. *M. scrofulaceum*, *M. nonchromogenicum* and *M. aurum* were isolated more frequently from Seoul soil samples than from Hiroshima soil samples, but *M. thermoresistibile*, *M. smegmatis* and *M. phlei* were isolated more frequently from Hiroshima soil samples. Interestingly, no *M. chelonei subsp. chelonei* observed in Hiroshima soil samples were isolated from Seoul soil samples.

Experimental atypical mycobacteriosis in mice; *M. scrofulaceum* and ten strains of AM were selected to compare the experimental atypical mycobacteriosis to the mycobacterosis due to *M. tuberculosis* $H_{37}Rv$. Changes of body weight in each group of mice inoculated with selected atypical mycobacterial strains (Table 5) are shown in Figure 3. The body weight of mice increased in both groups, that is, untreated control group and AM inoculated group. Even though differences in the change of body weight of mice were dependent on which AM was inoculated, the increment of body weight was less in the AM inoculated group as compared to the increment in the untreated control group. In contrast to the increment of body weight in these two groups, the body weight in the *M. tuberculosis* $H_{37}Rv$ infected mice decreased. No mouse died spontaneously during the observation period.

Mice from each group were sacrificed after

Table 4. Identification of acid-fast bacilli (AFB) according to the physiological and biochemical properties

Area / Species \ Media	Hiroshima					Seoul				
	Ogawa		L-J	ATS	Total (%)	Ogawa		L-J	ATS	Total (%)
	1%	3%				1%	3%			
M.* tuberculosis	0	0	0	0	0	0	0	0	0	0
M. bovis	0	0	0	0	0	0	0	0	0	0
M. scrofulaceum	3	3	3	2	11 (6.4)	6	5	2	5	19 (11.6)
M. gordonae	2	2	2	0	6 (3.5)	1	0	0	1	1 (1.2)
M. intracellulare	0	0	0	0	0	0	0	0	0	0
M. terrae	1	0	0	0	1 (0.6)	0	1	1	0	2 (1.2)
M. nonchromogenicum	3	3	1	0	7 (4.1)	5	3	3	1	12 (7.3)
M. fortuitum	19	16	14	10	59 (34.3)	20	18	19	11	68 (41.5)
M. fortuitum (thermophilum)	4	2	2	2	10 (5.8)	4	2	2	2	10 (6.1)
M. smegmatis	3	5	2	3	13 (7.6)	2	0	1	1	4 (2.4)
M. aurum	1	1	1	1	4 (2.3)	3	2	3	2	10 (6.1)
M. agri	3	3	2	2	10 (5.8)	2	2	2	2	8 (4.9)
M. cheronei subsp. chelonei	2	1	0	0	3 (1.7)	0	0	0	0	0
M. thermoresistibile	4	4	5	5	18 (10.5)	1	1	2	1	5 (3.0)
M. phlei	3	5	2	3	13 (7.6)	1	1	1	0	3 (1.8)
Unidentifiable strain	5	3	5	4	17 (9.8)	6	5	7	3	21 (12.8)
Total	53	48	39	32	172 (100.0)	51	40	43	30	164 (100.0)

* M: Mycobacterium,

a certain period of time and the gross changes of lung, spleen, liver and kidney are shown in Table 6. In the *M. tuberculosis* $H_{37}Rv$ inoculated group, splenic enlargement and small nodules in the spleen appeared on the second week after inoculation.

Pathological changes in other organs, also, were observed and these changes became progressively more severe with time. In the inoculated group, a few nodular changes in spleen and liver were observed in the first week after inoculation of *M. chelonei subsp. chelonei*, while enlargement of organs, congestion and nodular changes were observed in the second week in *M. scrofulaceum*, *M. agri*, *M. smegmatis*, *M. fortuitum*, *M. thermoresistibile*, and *M. nonchromogenicum* inoculated groups. Pathological changes started to subside from the third week in *M. chelonei subsp. chelonei* inoculated group and these changes had completely subsided

Table 5. Strains tested and viable units of inoculum to nice

Strains	Area of Isolation	Viable unit of Inoculum
M.* scrofulaceum	Seoul	2.8×10^8
M. gordonae	Hiroshima	3.2×10^7
M. agri	Hiroshima	5.4×10^8
M. smegmatis	Hiroshima	2.6×10^7
M. aurum	Seoul	6.4×10^8
M. fortuitum	Seoul	8.2×10^7
M. terrae	Hiroshima	7.4×10^7
M. chelonei subsp. chelonei	Hiroshima	4.4×10^8
M. thermoresistibile	Hiroshima	3.0×10^8
M. nonchromogenicum	Seoul	6.6×10^8
M. tuberculosis $H_{37}Rv$	Hiroshima Univ.	3.2×10^6

* M : Mycobacterium

by six weeks in groups inoculated with other AM. In all experimental groups but the *M. tuberculosis* $H_{37}Rv$ inoculated group, pathological changes subsided spontaneously. However, no gross pathological changes were observed in *M. gordonae*, *M. aurum*, and *M. terrae* inoculated groups.

Root index of lungs and spleen ; These indices were derived from the changes of organ weight and these are shown in Table 7. A ten or higher root index of the lungs and a one or higher root index of the spleen were considered significant changes. A ten or higher root index of the lungs was obtained after three weeks in the *M. tuberculosis* $H_{37}Rv$ inoculated group and in the *M. scrofulaceum* inoculated group and this index started to decrease after the sixth week in the *M. scrofulaceum* inoculated group.

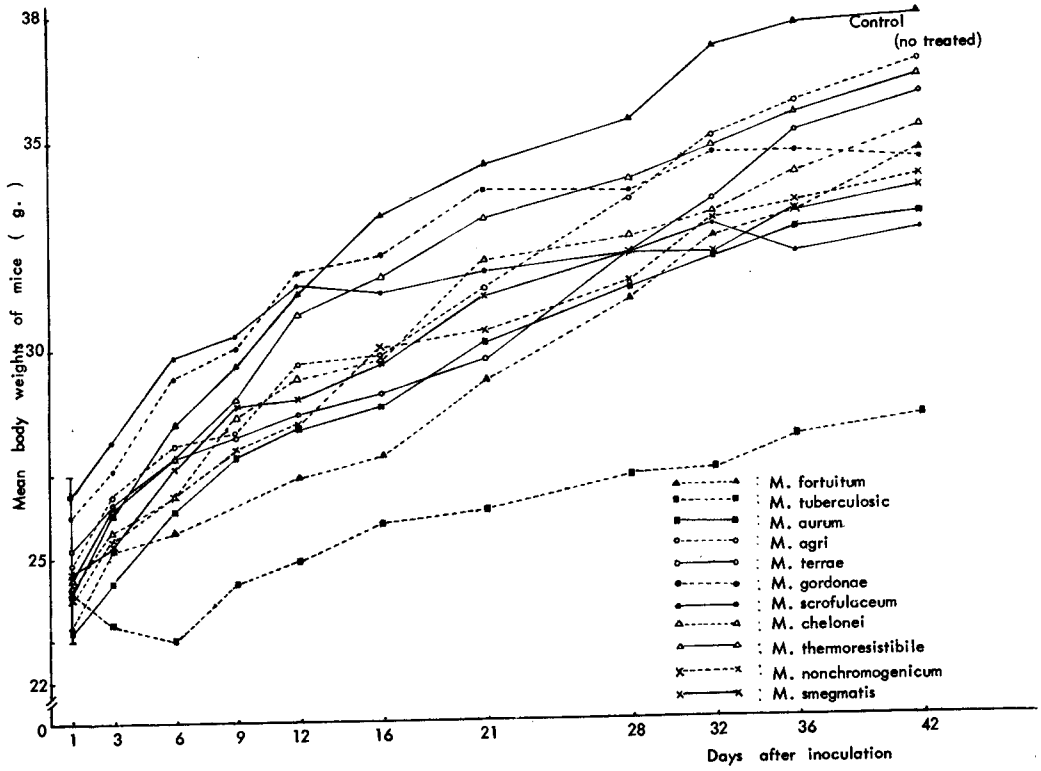

Fig. 3. Mean body weight after inoculation with mycobacterium species.

Table 6. Macroscopic findings of internal organs of mice at autopsies

Strains Tested	M.* scrofulaceum				M. gordonae				M. agri				M. smegmatis				M. aurum				M. fortuitum			
Weeks \ Organs	Lu.	Sp.	Li.	Ki.	Lu.	Sp.	Li.	Ki.	Lu.	Sp.	Li.	Ki.	Lu.	Sp.	Li.	Ki.	Lu.	Sp.	Li.	Ki.	Lu.	Sp.	Li.	Ki.
1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
2	−	++	++	−	−	−	−	−	−	+	+	−	−	+	+	+	−	−	−	−	−	++	++	+
3	−	++	++	+	−	−	−	−	−	++	+	−	−	+	+	−	−	−	−	−	−	++	++	++
4	++	++	++	−	−	−	−	−	−	−	−	−	−	+++	++	−	−	−	−	−	−	+	+	+
5	++	+	+	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−
6	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Strains Tested	M. terrae				M. chelonei				M. thermoresistibile				M. nonchromogenicum				M. tuberculosis $H_{37}Rv$				Control Not treated			
Weeks \ Organs	Lu.	Sp.	Li.	Ki.	Lu.	Sp.	Li.	Ki.	Lu.	Sp.	Li.	Ki.	Lu.	Sp.	Li.	Ki.	Lu.	Sp.	Li.	Ki.	Lu.	Sp.	Li.	Ki.
1	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
2	−	−	−	−	+	++	++	−	−	++	+++	−	+	+	+	−	−	+	+	−	−	−	−	−
3	−	−	−	−	−	−	−	−	+	+	++	+	+	++	+	−	+	++	+	−	−	−	−	−
4	−	−	−	−	−	−	−	−	+	+	++	+	−	++	+	+	++	++	+	+	−	−	−	−
5	−	−	−	−	−	−	−	−	+	+	++	−	−	+	−	−	+++	+	−	−	−	−	−	−
6	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+++	−	−	−	−	−	−	−

Lu. : Lungs, Sp. : Spleen, Li. : Liver, Ki. : Kidneys * M : Mycobacterium
− : No macroscopic findings. ± : Questionable lesions, + : Several small nodules. ++ : Less than 30 nodules
+++ : More than 30 nodules.

A higher than one root index of the spleen was obtained between three and four weeks in the *M. fortuitum* and *M. tuberculosis* $H_{37}Rv$ inoculated groups. However, it decreased to less than first after the fifth week in both groups. *M. tuberculosis* $H_{37}Rv$ inoculated group showed higher than one root index of spleen between three and six weeks but no other AM inoculated groups showed significant changes in the splenic index.

Re-isolation of AFB from organs ; Table 8 shows the results of re-isolation of AFB from the organs of the mice. *M. gordonae, M. aurum* and *M. terrae* were not re-isolated from organs of mice injected with the appropriate mycobacteria in the first week after inoculation *M. scrofulaceum* was re-isolated from the spleen of mice inoculated originally with this mycobacteria and in the second week *M. smegmatis, M. fortuitum, M. chelonei subsp. chelonei, M. thermoresistibile* and *M. tuberculosis* $H_{37}Rv$ were re-isolated from spleen, liver and kidney in mice inoculated originally with these microorganisms. However, re-isolation of AFB from mouse organs except those inoculated with *M. tuberculosis* $H_{37}Rv$ was not possible 5 and 6 weeks after inoculation. It was possible to re-isolate *M. agri, M. chelonei subsp. chelonei,* and *M. nonchromogenicum* from spleens and livers on the third and fourth weeks of inoculation in mice inoculated with the appropriate AM.

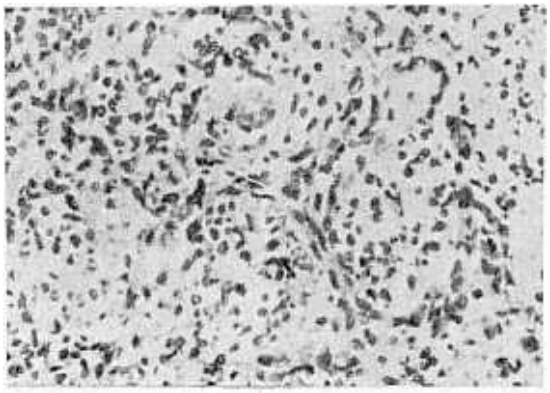

Fig. 4. Spleen, 4 weeks after inoculation with *M. scrofulaceum* Note. Nectrotic lesion (X240, H-E)

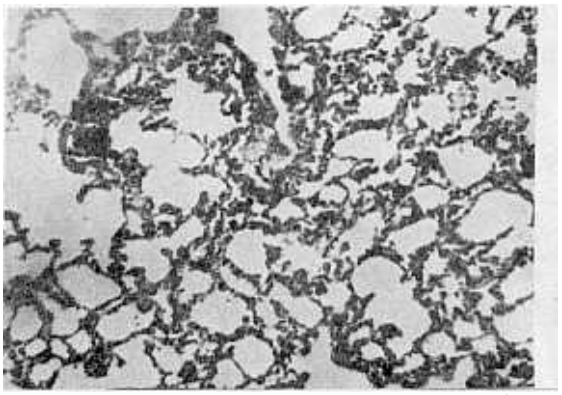

Fig. 5. Lung, 4 weeks after inoculation with *M. fortutium.* Note. Alveolar wall thickening (X120, H-E)

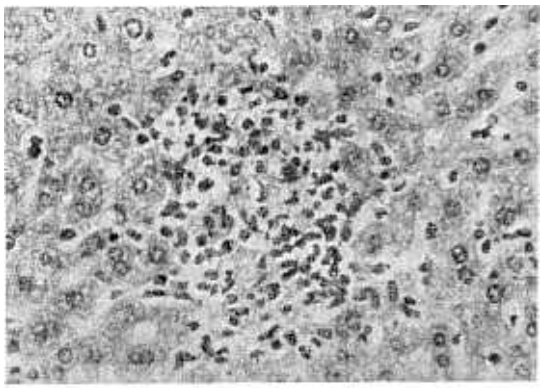

Fig. 6. Liver, 4 weeks after inoculation with *M. scrofulaceum.* Note. Necrotic changes (X240, H-E)

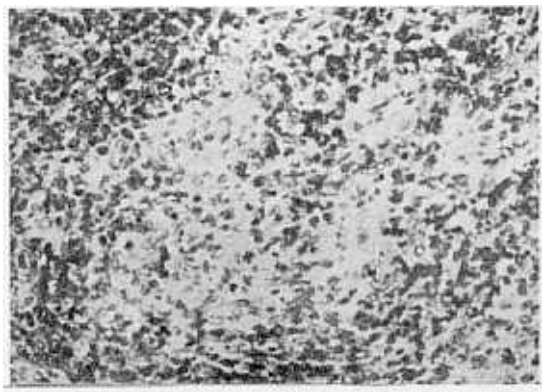

Fig. 7. Kidney, 4 weeks after inoculation with *M. nonchromogenicum.* Note. Tuberlar distriuction and non specific inflammation (X240, H-E)

Histopathological changes ; Table 9 shows the results. Mononuclear cell infiltration in lungs, spleen, and liver, alveolar wall thickening of lungs, and necrotic changes in spleen(Figure 4,5,6, and 7) were detected on the fourth week in *M. scrofulaceum*, *M. smegmatis, M. fortuitum*, and *M. nonchromogenicum* inoculated groups. After the sixth week, all these pathological changes subsided. In the *M. tuberculosis* $H_{37}Rv$ inoculated group, granulomatous changes in the liver in the fourth week and in the lungs in the sixth week of inoculation were observed.

In vitro sensitivity tests of re-isolated AFB to anti-mycobacterial agents; In vitro sensitivity tests of AM, *M. scrofulaceum, M. agri, M. smegmatis, M. fortuitum, M. chelonei subsp. chelonei, M. thermoresistibile,* and *M. nonchromogenicum;* and *M. tuberculosis* $H_{37}Rv$ as a control group to anti-mycobacterial agents (PAS, SM, INH, TH, CS, EB, and RFP) are shown in Table 10. *M. tuberculosis* $H_{37}Rv$ was sensitive to the anti-mycobacterial agents used but seven strains of AM were resistant to PAS, SM, and INH. *M. scrofulaceum, M. smegamatis,* and *M. nonchromogenicum* were moderately sensitive to TH and *M. nonchromogenicum* was moderately snsitive to CS. Growth of *M. fortuitum* and *M. chelonei subsp. chelonei* was not inhibited in all three different concentrations of EB and only the highest concentration of EB could inhibit the growth of

Table 7. Root indices of weight with lungs and spleen

Mycobacterial species	Organs	Root indices after inoculation (Weeks)					
		1	2	3	4	5	6
M. scrofulaceum	Lungs*	8.4	8.7	10.2	10.1	10.4	9.3
	Spleen**	0.72	0.89	1.31	1.02	0.81	0.79
M. gordonae	Lunngs	7.4	7.9	7.7	7.3	7.1	7.2
	Spleen	0.71	0.67	0.69	0.86	0.65	0.66
M. agri	Lungs	7.2	8.1	8.1	9.6	7.2	7.0
	Spleen	0.69	0.74	0.91	0.93	0.81	0.70
M. smegmatis	Lungs	7.9	7.9	7.7	7.1	6.4	6.2
	Spleen	0.72	0.77	0.92	0.86	0.81	0.68
M. aurum	Lungs	7.7	7.6	7.7	7.3	7.1	7.0
	Spleen	0.68	0.63	0.76	0.71	0.67	0.69
M. fortuitum	Lungs	8.1	9.4	9.5	9.6	8.4	7.0
	Spleen	0.72	1.01	1.20	1.13	0.79	0.62
M. terrae	Lungs	7.2	7.9	7.4	7.4	7.1	6.8
	spleen	0.62	0.64	0.64	0.65	0.68	0.67
M. chelonei subsp. chelonei	Lungs	8.1	8.9	7.7	7.6	7.4	7.1
	Spleen	0.93	0.99	0.87	0.90	0.84	0.88
M. themoresistibile	Lungs	7.9	9.2	9.2	9.7	8.1	8.1
	Spleen	0.77	0.84	0.81	0.80	0.82	0.80
M. nonchromogenicum	Lungs	8.2	8.9	8.9	8.3	8.0	8.2
	Spleen	0.76	0.92	0.97	0.98	0.91	0.88
M. tuberculosis	Lungs	7.3	9.0	10.8	11.2	12.6	12.4
(H_{37} Rv)	Spleen	0.77	0.91	1.12	1.40	1.31	1.25
Control	Lungs	7.8	7.6	7.7	7.2	7.3	7.9
(Not treated)	Spleen	0.67	0.61	0.69	0.70	0.68	0.69

* : Root Index of Lungs = $\sqrt{\frac{\text{Lung weight (mg)}}{\text{Body weight (g)}}} \times 10$ **: Root Index of Spleen = $\sqrt{\frac{\text{Spleen weight (g)}}{\text{Body weight (g)}}} \times 100$

M. thermoresistibile, *M. nonchromogenicum*, and *M. scrofulaceum*. *M. agri*, *M. chelonei subsp. chelonei*, and *M. tuberculosis* $H_{37}Rv$ were sensitive to all the concentration of RFP. *M. scrofulacrum*, *M. smegmatis*, *M. fortuitum*, and *M. thermoresistibile* were resistant to all three different concentration of RFP.

Immunodiffusion with AM antigens and sera from patients with leprosy ; Agar gel immunodiffusion was performed between sera of 29 patients with leprosy and a number of AFB antigens(Table 11). Twenty there sera (79.3%) showed positive precipitation reaction to *M. scrofulaceum* antigen. Only 5 sera(17.2%) exhibities positve reaction to *M. nonchromogenicum*.

All sera samples were grouped according to the type of leprosy ; lepromatous type(22) and tuberculoid type(7). Seventeen(77.3%) sera out of 22 sera from the lepromatous types were reactive to *M. scrofulaceum* and 14(63.6%) out of 22 sera to *M. gordonae*. Out of 7 sera from the patients with tuberculoid type, 5 sera(71.4%) were reactive to *M. scrofulaceum*,

Table 8. Mean viable units recovered from 50 mg. of each organ of mice

Strains Tested \ Observation: Organ	Weeks after inoculation																							
	1				2				3				4				5				6			
	Lu.	Sp.	Li.	Ki.	Lu.	Sp.	Li.	Ki.	Lu.	Sp.	Li.	Ki.	Lu.	Sp.	Li.	Ki.	Lu.	Sp.	Li.	Ki.	Lu.	Sp.	Li.	Ki.
M. scrofulaceum	−	±	−	−	−	+	+	−	+	+	+	+	−	+	+	−	+	+	+	−	−	−	−	−
M. grodonae	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
M. agri	−	−	−	−	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	−	−	−	−	−
M. smegmatis	−	−	−	−	−	++	++	−	−	++	++	−	−	++	++	−	C	+	+	−	−	−	−	C
M. aurum	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
M. fortuitum	−	−	−	−	−	+	++	++	−	+	++	++	−	+++	++	++	−	+	±	+	−	−	−	−
M. terrae	−	−	−	−	−	−	−	−	−	−	−	C	−	−	−	−	−	−	−	−	−	−	−	−
M. chelonei subsp. chelonei	−	−	−	−	−	++	++	−	−	++	++	−	−	+	+	−	−	−	−	−	−	−	−	−
M. thermoresistibile	−	−	−	−	−	−	±	−	−	+	++	+	−	++	++	−	−	C	+	C	−	−	−	−
M. nonchromogenicum	−	−	−	−	−	−	−	−	−	+	+	−	−	++	++	−	−	−	−	−	−	−	−	−
M. tuberculosis $H_{37}Rv$	−	−	−	−	−	+	±	−	+	++	++	+	++	+++	+++	+	++	+++	+++	+	++	+++	+++	+
Control	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	C	−	−	−	−	−	−	−	−

Lu : Lung, Sp : Spleen, Li : Liver, Ki : Kidneys
− : No growth, ± : < 10 colonies, + : 10 - 30 colonies, +± : 31 - 50 colonies, ++ : 51 - 80 colonies, +++ : > 80 colonies, C : contamiation

Table 9. Pathological findings after inoculation with mycobacterial species

Strains Tested \ Observation: Organs	Weeks after inoculation									
	4					6				
	Lu.	Sp.	Li.	Ki.	Ly.	Lu.	Sp.	Li.	Ki.	Ki.
M. scrofulaceum	+	++	++	−	−	−	−	−	−	−
M. gordonae	−	−	−	−	−	−	−	−	−	−
M. agri	−	−	−	−	−	−	−	−	−	−
M. smegmatis	+	+	+	−	−	−	−	−	−	−
M. aurum	−	−	−	−	−	−	−	−	−	−
M. fortuitum	++	+	+	+	−	−	−	−	−	−
M. terrae	−	−	−	−	−	−	−	−	−	−
M. chelonei subsp. chelonei	−	−	−	−	−	−	−	−	−	−
M. thermoresistibile	−	+	−	−	−	−	−	−	−	−
M. nonchromogenicum	−	+	++	−	−	−	−	−	−	−
M. tuberculosis $H_{37}Rv$	++	++	+++	+	++	+++	++	+++	++	++
Control	−	−	−	−	−	−	−	−	−	−

Lu : Lung, Sp : Spleen, Li: Liver, Ki : Kidneys, Ly : Lymphnode
− : No changes, + : nonspecific inflammation, ++ : thickening or necrosis, +++ : granuloma

Table 10. Sensitivities to antituberculosis drugs of mycobacteria reisolated from the mice experimental groups

Strains Tested \ Concentration of Drugs (mcg/ml)	PAS			SM			INH			TH			CS			EB			RFP			Control
	5	10	20	10	100	200	5	10	20	25	50	100	20	40	80	5	10	20	25	50	100	
M.* scrofulaceum	+++	+++	+++	++	++	++	++	++	++	++	++	−	++	+	±	+	−	−	+++	+++	++	+++
M. agri	++	++	++	+	+	+	++	+	+	+++	++	++	++	++	+	++	−	−	−	−	−	+++
M. smegmatis	+++	+++	+++	++	++	+	++	+	+	+	±	−	++	++	++	−	−	−	++	++	+	+++
M. fortuitum	+++	+++	+++	++	++	++	++	+	+	+++	++	+	+++	+++	+++	++	++	+	++	++	++	+++
M. chelonei subsp. chelonei	++	++	++	+++	++	+	++	++	++	++	++	+	++	+	+	+	+	+	−	−	−	+++
M. thermoresistibile	++	++	++	+	+	+	+	+	+	++	++	++	+	+	+	++	+	−	++	++	+	+++
M. nonchromogenicum	++	++	++	+	+	+	+	+	+	+	−	−	++	+	−	++	+	−	++	−	−	+++
M. tuberculosis $H_{37}Rv$	++	+	−	−	−	−	+	−	−	++	−	−	−	−	−	−	−	−	−	−	−	+++

PAS : para-aminosalicylic acid, SM : streptomycin, INH : isonicotinic acid hydrazide TH : ethionamide, CS : cycloserine, EB : ethambutol, RFP : rifampicin,

* M: Mycobacterium

− : No growth, ± : less than 20 colonies, + : 21 - 50 colonies, ++ : 51 - 100 colonies, +++ : numerous colonies

Table 11. Precipitation reaction using 29 leprosy sera against various mycobacterial antigens by agar gel immunodiffusion

Antigen	Positive	Type/case		Bacteriology/case	
		L/22	T/7	Pos./18	Neg./4
*M.*agri*	8(27.5)	6(27.3)	2(28.6)	NT	NT
M. aurum	13(44.8)	11(50.0)	2(28.6)	6(33.3)	3(75.0)
M. gordonae	18(62.1)	14(63.6)	3(42.9)	10(55.6)	3(75.0)
M. nonchromogenicum	5(17.2)	5(22.7)	−(0.0)	3(16.7)	1(25.0)
M. scrofulaceum	23(79.3)	17(77.3)	5(71.4)	11(61.1)	3(75.0)

* M : Mycobacterium, − : No reaction, NT : Not tested
( ) : % of positive cases, L : Lepromatous type, T : Tuberculoid type

3 sera(42.9%) to *M. gordonae*, 2 sera(28.6%) to *M. agri* and *M. avium*. However, all seven of these sera were not reactive to *M. nonchromogenicum*. Twenty two lepromatous type patients were divided into two groups on the basis of the Wade skin scraping test; a bacteriologically positive group(18 cases) and negative group(4 cases). No siginficant differences in the precipitation reactions between bacteriologically positive and negative groups were observed.

DISCUSSSION

Numerous studies have established that AFB can be isolated from the natural environment (Kubica, *et al.*, 1961; Kubica, 1973, Mallman, *et al.*, 1962; 1963, Bailey *et al.*, 1970 Gruft *et al.*, 1976, Yamaoka, 1977). The present study was undertaken to compare the distributions of atypical mycobacteria in soil samples from Seoul, Korea and Hiroshima, Japan. The Söhngen method (Söhngen, 1913) and the Wolinsky method (Wolinsky and Rynearson, 1968) were employed in pre-treating the soil samples and four different solid media (1% Ogawa egg medium, 3% Ogawa egg medium, Löwenstein-Jensen medium, and American Trudeau Society medium) were used for the purpose of isolating AFB. Using 1% Ogawa egg medium, no differences were observed on the isolation rate of AFB from soil samples treated by the two pretreatment methods; i.e. 46.7% by Söhngen method and 44.2% by Wolinsky method from Seoul samples 45.8% by Söhngen method and 58.3% by Wolinsky method from Hiroshima samples. 1% Ogawa egg medium exhibited the highest isolation rate of AFB from soil samples. Five percent sulfuric acid solution used in Söhngen method was not strong enough to eliminate saprophytes in soil samples since most of the isolated AFB were rapid growers and no slow growers were isolated. Thus, it suggests that NaOH solution treatment is preferred to H_2SO_4 treatment in order to isolate AFB from soil. Cycloheximide and NaOH solution treatment used in the Wolinsky method were effective in eliminating saprophytes. Nevertheless, this method is more complicated than the Söhngen method samples have to be treated with 4% NaOH and 1N-HCl is used to isolate *M. tuberculosis*.

It was not possible to sub-culture thirteen strains of 349 strains isolated from the samples (Table 2). This result suggests that fastidious acid-fast organisms, which need such complex culture conditions that it is not possible to culture them in artificial media, might exist and further studies will be necessary to resolve this

question. Most of the isolated *in vitro* cultivable AFB from both regions, applying the Söhngen method, were classified as rapid growers of the Runyon groups (Table 2). Kubica and Vestal (1961), Kubica *et al.* (1963) and Jefferies *et al.* (1963) reported that scotochromogens and nonphotochromogens were the most frequently isolated AFB from natural environment and rapid growers were the next most frequently isolated. The difference between their reports and the result of this study suggests that the distribution of AFB in the natural environment might differ from region to region. Four strains out of 25 slow growers from Hiroshima soil samples and 5 strains out of 35 slow growers from Seoul soil samples were low temperature sensitive strains which did not grow at 28°C. Seventeen strains out of 147 rapid growers from Hiroshima soil samples and 21 out of 129 rapid growers from Seoul soil samples were classified as unidentifiable species of AFB as their biological and biochemical characteristics did not match with those of standard AFB strains of Saito (1975) and Tsukamura(1975). Some regional differences between Seoul and Hiroshima in the distribution of AFB strains could be observed; higher number of slow growers (especially *M. scrofulaceum* among them) were isolated from Seoul soil samples than from Hiroshima soil samples. No *M. tuberculosis*, *M. bovis*, *M. avium*, and *M. intracellulare* were isolated from the soil of either region. However, *M. fortuitum* was isolated from the soil of both regions. *M. ulcerans*, *M. kansasii*, *M. marinum*, *M. avium*, *M. intracellulare*, *M. fortuitum*, *M. chelonei subsp. chelonei*, and *M. chelonei subsp. abscessus*, *M. simiae*, and *M. shimoidei* have been reported to be pathogenic to humans. Recently, Tsukamura(1977) reported that *M. gordonae*, *M. triviale*, and *M. nonchromogenicum* which previously had been known as non-pathogenic to human have now behaved as pathogens to human.

Ten different AM strains among 338 AM which were isolated from soil samples of both regions and which showed a high isolation rate or regional differences were selected and inoculated into ddY mice to observe the experimentally induced atypical mycobacteriosis. Timpe and Runyon(1954) observed that after intravenous inoculation of 0.2 mg photochromogens, lesions occurred mainly in lungs and that this dose was lethal to mice, but no lesions were observed after the inoculation of scotochromogens. Engbaek (1961) reported that lymphocyte infiltration and epitheloid nodules were observed in lesions after intravenous inoculation into mice of 1 *mg*, 0.01 *mg* and 0.0001 *mg* of four photochromogenic strains. In the present study, all mice were inoculated intravenously with 0.2 *mg* of AM ; all the inoculated survived during the six weeks observation period. The body weight of mice injected with AM isolated from soil increased less than the body weight increments of untreated control mice. However, no difference was observed between different AM inoculated mice groups. Thus, it was not possible to evaluate the virulence of AM according to the change of body weight. The data presented in this experiment is similar to that of Kuze *et al.* (1978). Significant increases of lung root indices were observed in the group inoculated with *M. scrofulaceum* and *M. fortuitum* inoculated groups, but these increases did not persist. Gross changes (Table 6) and, histopathological changes(Table 9 and Figure 4,5,6,7) of organs and the reisolation of AM from infected organs were also transient. Each AM exhibited differences in the peristency of lesions in mice and the multiplication and invasion of AM in inoculated mice was limited.

However, these changes persisted in mice

inoculated with *M. tuberculosis.*

A number of investigators have reported the resistancy of AM to antimycobacterial agents in the *in vitro* sensitivity test of AM to anti-mycobacterial agents(Runyon, 1959; Leach and Fenner, 1954; Wolinsky, 1959; Manten, 1959; Virtanen, 1961; Nakamura, 1964; Nagusa, 1968; Rynearson *et al.*, 1971; Kajioka and Hui, 1978; Naito *et al.*, 1979). Some of the AM re-isolated from infected organs exhibited moderate sensitivity to TH, EB, CS, and RFP but most of the AM were resistant to anti-mycobacterial agents (Table 10) and were more resistant than AM isolated from human. Among the isolated AM, *M. agri, M. smegmatis, M. thermoresistibile* and *M. nonchromogenicum,* exhibited mild degree of atypical mycobacteriosis.

Common antigens between different AM have been reported(Abe, 1970; Kim and Lew, 1972; Ridell and Norlin, 1973; Closs and Kronvall, 1975). In an attempt to observe common antigens between *M. leprae,* which at present cannot be cultivated *in vitro,* and AM from soil, agar gel immunodiffusion was performed with leprosy sera as antisera and AM isolated from soil as antigens. *M. scrofulaceum* antigen showed positive precipitation reactions with the highest number of leprosy sera (79.3%) and the lowest number(20.6%) of positive reactions was seen with *M. nonchromogenicum* antigen. It is quite possible therefore that the leprosy sera contains antibodies against other AFB. However, it probably contains antibodies mostly against *M. leprae.* If this is the case, it is possible that *M. scrofulaceum* shares a greater number of common antigens with *M. leprae* than other AM.

Since *M. tuberculosis* $H_{37}Rv$, *M. smegmatis, M. kansasii, M. lepraemurium* and *M. avium,* which have been reported as having a high number of common antigens with *M. leprae.* (Sushida and Hirano, 1961; Rees *et al.*, 1965; Navalker, 1971; Kim and Lew, 1972); were not included in this study, it is not possible to conclude that *M. scrofulaceum* has more common antigens with *M. leprae* than these previously reported mycobacteria. A higher percentage of positive precipitation reaction was observed with sera from lepromatous type patients, in whom *M. leprae* can be detected more easily and are clinically malignant, than with sera from tuberculoid type patients. This result could be due to the high level of immunoglobulins in the lepromatous type, where cell-mediated immunity is suppressed but humoral immunity is intact or enhanced, compared to the immunoglobulin level in the tuberculoid type. Sera from lepromatous type patients who had different bacterial indices did not show any signficance in the percentage of positive precipitation reactions.

Runyon(1959) assumed that mutated AFB could occur from natural environments where pathogenic and non-pathogenic AFB co-existed and these mutated AFB could cause diseases to human after establishing relationships with human as either symbiotic or parasitic inhabitants. Tarshis(1958) and Xalabardar(1961) assumed that the change of non-pathogenic AM into pathogenic AM could be a result of the use of anti-mycobacterial agents. Kubica *et al.* (1963) also suggested that these pathogenic came from soil, reasoning as follows; firstly, the incidence of AM infection is higher in rural areas than in cities, secondly, the distribution of atypical mycobacteriosis is sporadic, thirdly, not a single case of direct infection from human to human has been reported and fourthly, biological and biochemical characteristics of these AM isolated from patients are identical to those of AFB isolated from soil. Wolinsky and Ryneason(1968), also, mentioned the possibility of soil origion of pathogenic AM and Tsukamura(1977) reported that atypical myco-

bacteriosis was a kind of opportunistic infection affected by the general conditions of the host, the geometric distribution and virulence of the AM.

M. intracellulare and *M. kansasii* which are known to be pathogenic were not isolated from soil samples used in this study. The pathogenic changes due to AM, *M. scrofulaceum, M. fortuitum;* and *M. chelonei subsp chelonei* isolated in this study were transient and these lesion subsided spontaneously while those due to *M. tuberculosis* H_{37}Rv were progressive. The fact that some of the AM isolated in this study exhibited transient pathologic changes in mice suggests the possibility that atypical mycobacteriosis depend on the host-parasitic interaction.

CONCLUSION

In an attempt to study the relationship between AFB inhabiting in soil and atypical mycobacteriosis in human, the following experiments were performed, a) the effects of various treatment methods of soil and culture media on the isolation of AFB, b) geographical differences in distribution of AFB strains, c) the pathogenicity of these strains to mice, d) behavior of these strains to anti-mycobacterial agents, e) agar gel immunodiffusion with culture filtrates of AFB and sera from leprosy patients.

From these experiments, the following results were derived, 1) Söhngen method is preferable to the Wolinsky method for the isolation of rapid-growers and vice versa for the isolation of slow growers. Also, 1% Ogawa egg medium is the best medium for the isolation of AFB from soil. 2) No significant geometric differences between Hiroshima and Seoul soil samples in the distribution of AFB were observed. *M. fortuitum* is the AFB observed most frequently in soil samples from both regions and M. bovis, *M. avium-intracellulare* complex were not observed in soil samples of either regions. A larger number of *M. scrofulaceum* was isolated from Seoul soil samples than from Hiroshima's but *M. chelonei subsp. chelonei* which was isolated from Hiroshima soil samples was not observed in Seoul samples. 3) Rapid-growers are the most frequent AFB from soil, 147 strains(82.6%) from Hiroshima soil samples and 129 strains(75.4%) from Seoul soil samples. Seventeen strains(9.6%) of scotochromogen, 8 strains(4.5%) of nonphotochromogen were isolated from Hiroshima soil samples and 21 strains (12.3%) of scotochromogen, 14 strains(8.2%) of nonphotochromogen were isolated from Seoul soil samples. No photochromogen was isolated from soil samples of either region.

4) Fastiduious mycobacteria, which were siolated from soil but did not grow in subculture, were observed; 6 strains(3.3%) from Hiroshima soil samples and 7 strains(4.1%) from Seoul soil samples.

5) Transient atypical mycobacteriosis was observed in mice inoculated with *M. scrofulaceum, M. smegmatis, M. fortuitum, M. chelonei subsp. chelonei, M. thermoresitible* and M. *nonchromogenicum* but these mild pathological changes in organs subsided spontaneously.

6) Re-isolation of AFB between 2 and 6 weeks was possible in mice inoculated with *M. tuberculosis* $H_{37}Rv$. However, re-isolation of AFB from mice inoculated with other AFB including *M. gordonae, M. aurum,* and *M. terrae* was not possible.

7) All AFB strains re-isolated from organs of mice were resistant to PAS, SM, and INH. *M. scrofulaceum, M. agri, M. smegmatis, M. thermoresistibile,* and *M. nonchromogenicum* were sensitive to high concentrations of TH, CS, and EB. Strains sensitive to RFP were *M. agri* and *M. chelonei subsp. chelonei.*

8) *M. scrofulaceum* antigen showed a pre-

cipitation reaction in agar gel immunodiffusion with the highest number of sera from leprosy patients and *M. nonchromogenicum* antigens was the one which reacted with the least number of sera from leprosy patients. Bacterial index did not affect the frequency of positive precipitation reactions with AM antigens.

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