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ATTEMPTS AT GROWTH OF M. LEPRAE IN MICE AND OBSERVATIONS ON A CASE OF PROBABLE CONTAMINATION WITH ANOTHER ACID-FAST BACILLUS

Tatsuji Ogawa

Kitasato Institute and Urawa City Hospital

Our attempts to grow M. leprae in mice have been made, in the midst of studies of the cultivation of M. lepraemurium and in a rather unsystematic fashion, over the last four years. No experimental systems for the growth of M. leprae in susceptible animals had been established that would provide the large quantities of bacilli needed for cultural isolation. So, following previous workers we selected the mouse as an experimental animal and began a series of inoculation experiments by the subcutaneous and/or intravenous routes, the latter of infrequent use for such purposes. I want to describe the results of these experiments as well as the observations on a probable mycobacterial contamination encountered in one of the experiments.

MATERIALS AND METHODS

The technical procedures of experiment are explained briefly (Table 1). First, bacterial suspension prepared from biopsies of leprosy patients was injected into mice either once or several times (at weekly intervals) by the subcutaneous or intravenous route, or both. Secondly, the animals were killed at various intervals 2-16 months after injection. At necropsy lesions were sought by gross inspection. Fourthly, portions of various organs were removed and ground in a mortar to make a homogenate. Smears made from the homogenate were stained by Ziehl-Neelsen's method and examined microscopically. Lastly, the homogenate was treated with one percent sodium hydroxide solution and then inoculated onto both the egg yolk medium (for M. lepraemurium; also for M. leprae[?]) and Ogawa 1% egg medium (for cultivable mycobacteria except M. lepraemurium). The tubes were incubated at 37°C for over 3 months and examined at regular intervals macroscopically.

Table 2 shows the details of single inoculation experiments. The studies were based on three experiments with intravenous inoculation and another with intravenous and subcutaneous inoculation. Most of the animals employed were ddN strain and the rest were C3H strain, Inoculation was done with 0.1 or 0.2 ml of an inoculum. A solid circle indicates that in the experiment smear-positive animals were observed. Additional information about the number of mice used, the duration of survival, the number of times of autopsies, the origin of leproma, or the number of bacilli in inocula is shown in the table.

Table 3 shows the details of multiple inoculation experiments. These include four experiments with intravenous inoculation, one with intravenous and subcutaneous inoculation, and one with subcutaneous inoculation. Inoculum size varied from 0.1 to 0.5 ml, and in each animal inoculation was repeated 2-5 times at weekly intervals. A number mark in the Table indicates that the experiment showed a probable contamination with another acid-fast bacillus. A solid circle, as in Table 3, indicates the experiment with smear positive animals. More detailed information on the form and nature of experiments will be seen in the table.

Table 1. Outline of experimental procedures.

1. A suspension of M. leprae prepared from human leproma is inoculated either once or several times (at weekly intervals) into mice by the subcutaneous or intravenous route, or both.

2. The animals are sacrificed at different intervals during a period of 2 to 16 months after inoculation.

3. At necropsy macroscopic observations are made on the spleen, liver, lungs, kidneys and superficial lymph nodes.

4. A portion of organ removed is ground in a mortar to make a homogenate. Smears made from the homogenate are stained by Ziehl-Neelsen's method and examined microscopically.

5. After being treated with 1% sodium hydroxide solution, the homogenate is inoculated into Ogawa's egg yolk and egg media. The tubes are incubated at 37°C for over three months and examined visually for colonial growth at regular intervals.

RESULTS

1. Attempts at Growth of M. leprae in Mice. Altogether ten experiments were carried out; four with single inoculation and the other six with multiple inoculation. Because one experiment, Expt. (4), exhibited a probable contamination and its results are described in the following section separately, the results of the rest of the experiments will be mentioned here.

Gross findings were all negative. Cultural examination showed that no colonies of mycobacteria, suspected of being M. leprae have been isolated. A few smooth and buff colonies, supposedly atypical mycobacteria, were, however, isolated from two specimens.

On the other hand, microscopic examination revealed the presence of acid-fast bacilli in the tissues of various organs. Among the experiments, two, Expts. (3)-1 & -2, showed the most pronounced findings microscopically (Table 4). In both experiments, injection with a suspension of M. leprae was repeated 3 times. The animals were killed one by one at intervals from 2 to 11 months after inoculation. It will be seen that in the subcutaneous experiment bacillary counts for the injection site revealed Gaffky X, 2-3

months after injection. Thereafter, however, the bacilli decreased in number and could no longer be found after 9 months. Superficial lymph nodes scored Gaffky I during the first 4 months after inoculation. None of the tissues of viscera were smear-positive. On the other hand, in the intravenous experiment, Expt. (3)-2, acid-fast bacilli were detected in the spleen, liver and lungs, but smaller in number as compared with those at the injection site just mentioned. No bacilli were found in the superficial lymph nodes. In both of the experiments the acid-fast bacilli had a tendency to steadily decrease in number. Where numbers of bacilli were present, globi were often seen, but these were usually small in size and loose in arrangement. And, indeed, it is uncertain whether the bacilli had multiplied within the tissue or not.

Table 5 summarizes the microscopic findings obtained in all the experiments. The results are tabulated according to the form and route of injection. The data of single inoculation experiment showed that all the specimens obtained from animals injected intravenously were microscopically negative, but those from the animals simultaneously injected (iv + sc) were positive in 7 percent. In the case of multiple inoculation, the following percentages of smear-positive specimens were noted: intravenous, 13%; intravenous plus subcutaneous, 6%; and subcutaneous, 15%.

As the leproma used differed from experiment to experiment, it is impossible to compare the values of percentage for smear-positive specimens directly. As a whole, however, one seems fairly justified in concluding that the microscopic findings were better with multiple inoculation than with single inoculation. This fact is in accord with observations by previous workers.

2. Observations on a Case of Supposed Contamination with Another Acid-Fast Bacillus. The supposed contamination occurred in Expt. (4), the inoculation of which had been carried out by means of multiple intravenous injection (Table 6). The macroscopic, microscopic, and cultural examinations made 3 months after inoculation all gave negative results. At 12-month autopsy, however, the superficial lymph nodes exhibited varying degrees of macroscopic enlargement. One mouse (no. 8) revealed gross involvement also in the spleen, liver, and lungs. Microscopically, all the animals at this stage harbored acid-fast bacilli in the tissues of various organs such as spleen, liver, lungs, kidneys or pooled superficial lymph nodes; the tissues of mouse no. 8 had the heaviest bacillation. Culture results were as follows: Although nearly half of the tubes inoculated were contaminated with saprophytes; macroscopic, rough, and pale yellow-colored colonies could be isolated from the tissues of spleen and lungs of mouse no. 8. The primary growth occurred only on the egg yolk medium after 2 months' incubation.

When stained and examined microscopically, the isolates were found to be an acid-fast bacillus similar to M. tuberculosis. This is hereafter referred to as Bacillus No. 4. Its characteristics in comparison with those of M. lepraemurium are shown in the next slide (Table 7). The bacillus is very similar to M. lepraemurium in many respects, such as colony appearance, cell morphology, growth response on the egg and egg yolk media, growth temperature and niacin test.

On subculture, as in the case of M. lepraemurium, a small portion of colony of the preceding culture was transferred lightly on the middle of the surface of 1% egg yolk medium by loop, the tube of which was sealed with a rubber cap having silk threads allowing the entry of a little air. At present the fifth generation are growing. Table 8 indicates that the percentage of successful growth on subculture had no tendency to change with the age of the preceding culture or on transfer. In this regard, too, the bacillus resembled the control strain of M. lepraemurium in the 5th-8th generations. Measurements were made of the size of colonies to compare Bacillus No. 4 with M. lepraemurium in growth rate (Table 9). Both stains showed a very slow but steady growth till 5 months after inoculation and no significant differences were observed between them.

Reproduction of disease in mice was sought by inoculating Bacillus No. 4 in 0.1 mg amounts into C3H mice intravenously (Table 10). No macroscopic findings were obtained four and a half months after inoculation. After six months, however, marked involvement was observed in the spleen, liver, lungs and superficial lymph nodes macroscopically. The response patterns resemble those in mice infected with M. lepraemurium. Microscopic study revealed heavy bacillation in various tissues including lymph nodes 4 1/2 - 6 months after inoculation. On cultural isolation, mycobacterial colonies were recovered on the egg yolk media only from heavily bacillated tissues. The characteristics of the isolates were much the same as those of the original culture employed for inoculation.

The bacillus is now still under examination so that no final conclusion can be made as yet on its taxonomic position. Finally, I would like to present several additional slides showing the following pictures: a culture of Bacillus No. 4, its stained smear, histopathologic preparations made from the infected mice on primary isolation as well as on reproduction of disease.

DISCUSSION AND CONCLUSION

Our attempts resulted in a simple confirmation of the reports by previous workers. In a series of studies on cultivation of M. lepraemurium we have observed that there are many cultural properties common between M. lepraemurium and M. tuberculosis. In a similar sense, there might be the analogy between M. leprae and M. lepraemurium. With such a speculation I have made the above experiments in the hope of culturing M. leprae in vitro. Recently, successful infections of armadillo with M. leprae have been reported. If our method for culturing M. lepraemurium is applied to heavily bacillated specimens from such infected animals, one might perhaps some day pave the way to success in the cultivation of M. leprae.

Table 2. Details of single inoculation experiments for propagating M. leprae in mice.

Route[a]	Expt. no.[b]	Mice		Suspension of M. leprae			Duration of survival	Number of autopsies
		Strain	No.	Origin of leproma	Number of bacilli[c]	Inoculum[d]		
	(1)	ddN	9	A.O. ♀	V	0.2 ml x ①	2- 16 mth	5
iv	(2)-1	ddN	4	T.K. ♂	VII	0.2 ml x ①	2- 11 mth	2
	(8)-1	ddN & C3H	6	T.G. ♂	VI	0.1 ml x ①	7 mth	1
iv + sc	●(2)-2	ddN	4	T.K. ♂	VII	0.2 ml x ①	5- 8 mth	2

[a] iv, intravenous; sc, subcutaneous; iv + sc, simultaneously with each other.

[b] A solid circle(●) indicates that the experiment showed smear-positive animals.

[c] On the Gaffky scale.

[d] The numeral in open circle means the number of times of inoculations.

Table 3. Details of multiple inoculation experiments for propagating M. leprae in mice.

Route	Expt.[a] no.	Mice Strain	Mice No.	Suspension of M. leprae: Origin of leproma	Number of bacilli	Inoculum	Duration of survival	Number of autopsies
iv	●(3)-2	ddN	6	H.Y. ♀	X	0.2-0.3 ml x ③	2- 11 mth	6
	# (4)	ddN	5	T.K. ♀	II	0.2 ml x ④	3- 12 mth	2
	(6)	C3H	8	M.M. ♂	X	0.2 ml x ⑤	10- 13 mth	2
	●(8)-2	ddN & C3H	6	T.G. ♂	VI	0.1 ml x ⑤	7 mth	1
iv + sc	●(5)	ddN	8	I.K. ♂	VIII	0.1 ml x ②	6- 15 mth	3
sc	●(3)-1	ddN	6	H.Y. ♂	X	0.3-0.5 ml x ③	2- 11 mth	6

[a] A number mark(#) indicates the experiment with a probable mycobacterial contamination.
All footnotes of Table 2 apply also to this table.

Table 4. Two multiple-inoculation experiments with the most conspicuous microscopic findings.

Expt. no.	Mouse strain	Route	Duration of survival (months)	Viscera[a]				Inoculation site	Superficial lymph nodes
				spl.	lv.	lg.	kd.		
(3)-1	ddN	sc	2	0	0	0	0	X	I
			3	0	0	0	0	X	I
			4	0	0	0	0	II	I
			5	0	0	0	0	VIII	0
			9	0	0	0	0	0	0
			11	0	0	0	0	0	0
(3)-2	ddN	iv	2	II	II	IV	0	ne[b]	0
			3	II	0	0	0	ne	0
			4	0	0	0	0	ne	0
			5	II	II	0	0	ne	0
			9	I	I	0	0	ne	0
			11	I	I	0	0	ne	0

Roman numerals in the table represent bacillary scores on the Gaffy scale.

[a]Spl.=spleen, lv.=liver, lg.=lungs, kd.=kidneys.

[b]ne=not examined.

Table 5. Summary of results of microscopic examination.

Inoculation		Number of experiments	Number of mice	Viscera				Inoculation site	Lymph Nodes			Total
Form	Route			Spl	Lv	Lg	Kd		Knee	Axillary	Cervical	
"Single"	iv	3	19	0/19[b]	0/19	0/19	0/19	ne	0/8[c]			0/84(0%)
	iv + sc	1	4	0/4	0/4	0/4	0/4	ne	1/4	1/4	0/4	2/28(7%)
"Multiple"	iv	3*[a]	20	6/20	6/20	1/20	0/20	ne	0/20			13/100(13%)
	iv + sc	1	8	0/8	0/8	0/8	2/8	0/3	1/8			3/43(6%)
	sc	1	6	0/6	0/6	0/6	0/6	4/6	3/6	0/6	0/6	7/48(15%)

[a] Out of 4, one experiment(Expt. No.(4)) was excluded because of a probable contamination.

[b] No. Smear-positive/ No. Examined.

[c] Pooled lymph nodes.

Table 6. The one experiment with probable mycobacterial contamination.

Expt. No.	Mouse strain	Route & injections	Duration of Survival	Mouse no.	Gross Involvement[a] Spl	Lv	Lg	Kd	L-N	Number of Bacilli[b] Spl	Lv	Lg	Kd	L-N	Culture Results[c] Spl	Lv	Lg	Kd	L-N
(4)	ddN	iv; Five times	3 mth	3	-	-	-	-	-	0	0	0	0	0	.	.	.	.	.
				4	-	-	-	-	-	0	0	0	0	0	.	.	.	.	.
			12 mth	5	-	-	-	-	+~	I	0	0	I	I	x	x	ne	ne	.
				6	-	-	-	-	++~	IV	III	II	0	VI	x	x	ne	ne	x
				7	-	-	-	-	+~	0	0	0	I	I	.	.	.	.	.
				8	+	+	++	-	++~	X	X	IX	VII	X	◎	x	◎	x	.

[a] ++, moderate; +, sl. or trace; -, no change.

[b] On the Gaffky scale under oil-immersion(450x); L-N, lymph nodes.

[c] ◎, positive only on the egg yolk medium; ., negative; x, vitiated by contaminants; ne, not examined.

Table 7. Characteristics of Bacillus No.4.
i) Similarity to M. lepraemurium

	Bacillus No.4	M. lepraemurium strains Hawaii & Keishicho
1) Colony appearance:	Rough, pale yellow	Rough, pale yellow
2) Cell morphology and acid-fastness:	Similar to M. tuberc.	Similar to M. tuberc.
3) Growth on solid medium:		
a. 1% egg yolk medium	+	+
b. 1% egg yolk medium deprived of glycerol	-	-
c. Ogawa 1% egg medium	-	-
4) Growth temperature:		
40° C	-	-
37° C	+	+
30° C	+	+
22° C	-	-
5) Niacin test:	Negative	Negative

[a]1% egg yolk medium: Differs from Ogawa 1% egg medium only in containing the egg yolk instead of the whole egg; used for M. lepraemurium; pH 6.0- 6.2.

[c]Ogawa 1% egg medium: Used for M. tuberculosis; pH 6.6.

Table 8. Characteristics of Bacillus No.4
ii) Percentages of successful growth on subculture

	Age of the preceding culture transferred	Generation 2nd	3rd	4th	5th	Total
Bacillus No.4	2 mth	0/0[a]	15/18	0/0	3/3	18/21(86%)
	3 mth	13/14	4/11	0/0	5/6	22/31(71%)
	4 mth	0/0	0/0	2/2	0/0	2/2 (100%)
	Total	13/14 (93%)	19/29 (66%)	2/2 (100%)	8/9 (89%)	42/54 (78%)

	Age of the preceding culture transferred	Generation 5th	6th	7th	8th	Total
Strain Hawaii *M. lepraemurium* (Control)	2 mth	1/2	4/4	3/3	0/0	8/9 (89%)
	3 mth	4/5	5/7	1/1	4/4	14/17(82%)
	4 mth	2/2	3/4	8/8	4/4	17/18(94%)
	Total	7/9 (78%)	12/15 (80%)	12/12 (100%)	8/8 (100%)	39/44 (89%)

[a]No. Subculture-positive/No. Inoculated.

Table 9. Characteristics of Bacillus No. 4
iii) Size of colony growing on 1% egg yolk medium.

	No. of cultures followed up		Size of colonies (mm) after inoculation at 37° for			
			2 mo	3 mo	4 mo	5 mo
Bacillus No. 4	14	max-min	5-2	6-3	7-3	9-3
		average	3	4	5	6
Strain Hawaii M. lepraemurium (control)	20	max-min	5-2	6-3	8-4	10-4
		average	3	5	6	7

Table 10. Reproduction of disease in mice by Bacillus No. 4.

Culture used	Mouse Strain	Route & Inoculum	Duration of Survival	Mouse no.	Gross Lesion					Number of Bacilli					Culture Results				
					Spl	Lv	Lg	Kd	L-N	Spl	Lv	Lg	Kd	L-N	Spl	Lv	Lg	Kd	L-N
Bacillus No.4, 3rd sub-culture	C3H	iv; 0.1 mg	4½ mth	1	-	-	-	-	-	X	X	VIII	ne	VI	.	◎	.	ne	.
				2	-	-	-	-	-	VIII	X	X	ne	X	.	.	◎	ne	.
			6 mth	3	++	+++	++	-	++ to ++	X	X	X	IX	X	◎	◎	.	ne	◎
				4	+++	+++	+	-	++ to +++	X	X	X	VIII	X	◎	◎	.	ne	◎
				5	++	+++	++	-	++ to +++	X	X	X	X	X	x	.	◎	ne	◎