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Microscopic, Cultural and Serologic Studies on *Mycobacterium leprae* and other Mycobacteria Isolated from Leprosy Patients

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In a previous communication Olitzki and Godinger (4) reported the maintenance of *Mycobacterium leprae in vitro*. Uniformly stained bacteria were still present after nine passages on the medium described by Olitzki and Gershon (3), which, for short, will be called OG medium in the rest of this paper. The bacteria found after five months of incubation in the ninth passage were still uniformly stainable and did not show the bipolar staining described in degenerated bacteria by Rees and Valentine (5).

Since these experiments were carried out only with a strain originating from a patient who had acquired his infection in India, it seemed worthwhile to examine the possibility of maintaining other strains also of *M. leprae* for a prolonged period on the OG medium. The patients hospitalized in the Hansen Hospital in Jerusalem have come from different parts of the world, and acquired their infections in those regions. Twenty-six patients chosen for these examinations originated in the following countries: Afghanistan 1, Argentina 1, India 4, Iran 1, Iraq 3, Libya 1, Morocco 7, Palestine (British Mandate Territory) 1, Russia 1, Turkey 1, and Yemen 5. Three other patients under ambulatory treatment (Nos. 16, 17 and 22) were also chosen for these examinations, but did not appear on the dates for which the preparations for culturing were made. It seemed desirable first to determine if the uniform stainability of *M. leprae* originating in different countries is maintained equally well under the same *in vitro* conditions. Also, experiments were undertaken to determine whether strains originating from different countries are antigenically identical or whether antigenic differences occur.

MATERIALS AND METHODS

Microscopic examination of specimens. Before inoculation of the specimens, suitable quantities were spread on glass slides, stained with hot carbolfuchsin, and examined microscopially. Twe methods of counterstaining were employed simultaneously, one with methylene blue and the other with 1 per cent picric acid. When small tissue fragments were present, they became nontranslucent after staining with methylene blue. Counterstaining with pic-

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ric acid enabled easier recognition of bacteria adherent to tissue fragments, which appeared as brilliant red rods on a yellow background.

Cultivation of bacteria. Infected material was taken from the ear lobe tissue and from the nasal mucosa of the patients and inoculated into OG medium. Very small quantities were sufficient for the maintenance of a microscopically visible population. From the ear lobe traces of the infected fluid on the end of the scalpel sufficed to obtain positive results. Although the material from the ear lobe was taken under supposedly sterile conditions, it did not always prove to be sterile, and *M. leprae* was overgrown by saprophytic microorganisms. It was more difficult to obtain *M. leprae* free from concomitant saprophytic microorganisms, from the nasal mucosa. After numerous preliminary experiments it was found that *M. leprae* could be obtained free from such microorganisms from the ear lobe if 0.01 per cent malachite green was added to the first culture. In order to secure *M. leprae* free from saprophytic bacteria from the nose a higher concentration of malachite green (0.04%), and a more prolonged incubation period, were necessary. When these conditions were fulfilled, *M. leprae* appeared in the second passage free from living saprophytic microorganisms. This procedure, however, did not exclude the growth of saprophytic mycobacteria. Four patients harbored saprophytic mycobacteria in the ear lobe skin, three of them without *M. leprae,* and one, as shown by repeated examinations, together with it.

Complement-fixation tests. After the harvest of sufficient quantities of bacteria from the second and third passages, complement-fixation tests were performed with patients' sera. The antigens were prepared as follows: The bacteria were washed by centrifugation three times and the sediment was resuspended in saline. The sediment was submitted to sonic disintegration for two minutes, and the entire product, containing a soluble extract and insoluble remnants of cell walls, was employed as antigen. Fifty per cent of the maximal non-anticomplementary dose was used as antigen. Anticomplementary reactions due to any of the reagents were excluded by preliminary control experiments and suitable controls of the hemolytic system, the antigens, and the sera. The results were noted after all the control tubes had shown complete hemolysis. A second reading was made after the tubes were kept overnight in the refrigerator; this procedure allowed complete sedimentation of the erythrocytes and recognition of traces of hemolysis.

RESULTS

The positive results obtained by repeated examinations of the ear lobe skin are summarized in Table 1. All nine patients showed the symptoms of lepromatous leprosy clinically. The majority were immigrants from different countries, where they had acquired their infections, and in the majority of the examinations performed during recent years the microscopic findings were positive. In the microscopic examinations preceding the culturing of the tissue material taken from the cutaneous tissues *M. leprae* was found in varying quantities. After a short cultivation of about two weeks, in several cases (Nos. 20, 21, 27 and 29) more bacteria were found in the culture than by direct microscopic examination of the specimen. Hanks ([2]) has explained such findings as due to the liberation of bacteria encased in intact cells after autolysis of the tissues. Not all bacterial strains were maintained equally well on the OG medium. In the first experiment only seven out of nine strains yielded cell populations still staining well after 245 days on the first passage, with succeeding maintenance in the second and third passages, while two strains (from patients No. 20 and 21) were no longer visible. When the experiment was repeated with cultures from eight patients, only six of them were maintained. This ability to persist for a long period on OG medium has to be ascribed partly to the density of the bacterial population in the initial inoculum, which was very low in the specimens from patients No. 18, 20 and 21.

Table 2 shows the results obtained from the nasal mucosa of nine patients. In five of them the microscopic findings and the

TABLE 1. *Results of combined microscopic and cultural examinations of ear lobe skin.*

Patient no.	Birth place	Previous examination M. only		Combined examinations									
				1st experiment						2nd experiment			
				M. specimen	C. 1st passage incubation (days)		C. 2nd passage incubation (days)		C. 3rd passage incubation (days)	M. specimen	C. 1st passage incubation (days)		C. 2nd passage incubation (days)
		Since	Positive results		12	245	17	227	115		30	225	120
10	India	1965	10/10	3	3	2	2	2	2	2	2	1	1
11	Morocco	1965	7/8	2	2	2	4	3	4	3	3	2	2
13	Morocco	1964	15/16	3	4	2	3	2	3	4	4	2	3
15	Morocco	1963	7/11	3	4	2	4	3	2	2	4	1	1
18	Morocco	1963	8/13	1	1	2	4	2	3	1	0	0	—
20	Yemen	1951	7/7	1	3	0	4	0	0	—	—	—	—
21	Iraq	1952	3/16	1	3	0	3	0	0	1	0	0	—
27	Argentina	1964	12/12	2	4	2	4	4	3	4	4	4	4
29	Morocco	1965	9/11	2	4	4	4	4	3	4	4	3	3

Incubation periods till transfer in the first experiments: 1st passage—12 days, 2nd passage—55 days; in the second experiment: 1st passage—105 days.
M. = Result of microscopic examination of the original specimen. C = Result of microscopic examination of the culture.
0 = No microorganisms.
1 = One single microorganism in more than 10 fields.
2 = 1-10 microorganisms/field.
3 = 10-30 microorganisms/field.
4 = Masses of microorganisms/field.
— = Examinations not carried out.
The nominators in the fourth column indicate the number of positive results and the denominators—the total number of examinations.

TABLE 2. *Results of combined microscopic and cultural examinations of the nasal mucosa.*

Patient no.	Birth place	Previous examination M. only		Combined examinations											
				1st experiment						2nd experiment					
				M. specimen	C. 1st passage incubation (days)				C. 2nd passage incubation (days)	M. specimen	C. 1st passage incubation (days)			C. 2nd passage incubation (days)	
		Since	Positive results		9	45	105	225	135		32	92	122	30	135
8	Yemen	1951	0/5	0	0	0	0	0	—	0	0	0	0	—	—
9	Iran	1952	0/32	0	0	0	0	0	—	0	0	0	0	—	—
13	Morocco	1964	15/16	1	4	4	4	2	3	1	4	4	2	4	4
15	Morocco	1964	8/13	0	0	0	0	0	—						
18	Morocco	1964	3/13	0	0	0	0	0	—						
19	Morocco	1963	1/6	0	0	0	0	0	—	0	0	0	0	—	—
21	Iraq	1953	2/16	1	1	2	2	2	2	1	3	3	3	3	3
27	Argentina	1964	10/12	3	3	3	3	3	4	3	3	3	3	4	4
29	Morocco	1965	8/11							3	3	3	3	3	3

Incubation periods till transfer in the first experiment: 1st passage 105 days; in the second experiment; 1st passage—92 days.
Remarks and abbreviations as in Table 1.

TABLE 3. *Negative results obtained in examinations of the ear lobe skin.*

Patient no.	Birth place	Previous examinations		Combined experiment C. + M.
		Since	Positive results	
1	Russia	1961	0/7	0
2	Libya	1964	5/12	0
3	Morocco	1962	0/6	0
4	Yemen	1959	0/10	0
5	Afghanistan	1954	0/24	0
7	Palestine	1957	0/15	0
14	Yemen	1952	0/31	0
16	Yemen	1957	0/13	0
23	Iraq	1957	0/13	0
24	Iraq	1954	0/19	0
25	India	1953	0/26	0
26	Turkey	1960	0/10	0
28	India	1961	0/8	0

Remarks and abbreviations as in Table 1.

TABLE 4. *Saprophytic mycobacteria isolated from the ear lobes of four patients.*

Patient no.	Birth-place	Previous examinations			Microscopic examination before culturing	Primary culture on OG medium		Growth after transfer to Peizer or Dubos medium
		Since	Ear lobe	Nose		Direct	1st subculture	
8	Yemen	1951	1/5	0/5	0	3	3	4
9	Iran	1952	0/32	0/32	0	1	4	4
12	India	1965	8/8	7/8	3	4	4	4
19	Morocco	1963	1/6	1/6	0	3	4	4

Remarks and abbreviations as in Table 1.

results of the culture were negative. In four patients positive results were obtained microscopically and by culture as well. In two patients (Nos. 13 and 21), although only sporadic bacteria were found in the original specimen, dense bacterial populations appeared repeatedly in the culture. These findings were similar to those obtained in two instances with the bacteria from the ear lobe skin.

Table 3 shows the negative results obtained repeatedly on examination of the ear lobe skin of 13 patients. All of them, with the exception of No. 2, had been under treatment for many years, and the results of their microscopic examinations were consistently negative. Clinically, the majority of the patients showed the symptoms of lepromatous leprosy. Patients No. 3, 26 and 28 exhibited an indeterminate type of the disease, No. 4 the tuberculoid type, and No. 5 the neural type.

If we summarize the observations of the combined microscopic and cultural examinations, the following conclusions can be drawn:

(1) In all cases where bacteria were found microscopically, the initial cultures were positive and in some instances even showed higher population densities than the original specimens.

(2) Prolonged maintenance of stainable bacteria in subcultures was possible in only 13 out of 17 examinations of the ear lobe skin and in seven examinations of the microscopically positive nasal mucosa.

(3) In no case were positive cultures obtained from microscopically negative specimens.

Table 4 shows the results obtained in four cases in which atypical mycobacteria were isolated from the ear lobes. In three patients repeated microscopic examinations were negative. In one patient (No. 12), however, repeated microscopic examinations were positive. When additional cultures were made, mycobacteria appeared again, which were not cultivable on conventional media. That is to say, *M. leprae* was present without concomitant acid-fast microorganisms. Mycobacteria that are not identical with *M. leprae* have been isolated repeatedly from leprosy patients. Hanks (2) reported that *M. leprae* and rapidly growing mycobacteria were present at the same time in different nodules of the same patients. Similar results were published by Fraser and Fletcher (1).

All four strains isolated from the patients No. 8, 9, 12 and 19 were rapid growers. The intact bacteria and their cell walls were agglutinated by an anti-*M. fortuitum*-immune serum, strain 9 up to the end of the titer (1:3,250) and strains 8, 12, and 19 up to 1:625. Amidase reactions showed a pattern similar to that of *M. fortuitum*. The question arose whether the presence of these strains was due to accidental contamination by a laboratory strain or whether each patient harbored his own mycobacterium individually. Examination of the resistance of these strains showed that differences existed in their sensitivity to a group of antibiotics, a fact apparently justifying the conclusion that these strains originated from different sources.

Table 5 shows the behavior of these

TABLE 5. *The sensitivity of four rapidly growing mycobacterial strains isolated from leprosy patients to nine antibiotics.*

Strain No.	Antibiotic concentration (μgm./ml.)	Action of antibiotics no.								
		1	2	3	4	5	6	7	8	9
8	100.0	St	St	Bcd	0	St	St	Bcd	Bcd	St
(Yemen)	10.0	D	0	St	0	0	0	St	Bcd	0
	1.0	0	0	0	0	0	0	0	0	0
9	100.0	St	St	0	St	St	St	St	St	St
(Iran)	10.0	0	0	0	0	St	D	St	0	0
	1.0	0	0	0	0	St	St	St	0	0
	0.2	0	0	0	0	0	0	0	0	0
12	100.0	St	St	St	0	St	St	St	St	Bcd
(India)	10.0	St	0	St	0	D	St	St	St	0
	1.0	D	0	0	0	D	St	St	St	0
	0.2	D	0	0	0	D	0	0	D	0
	0.05	0	0	0	0	0	0	0	0	0
19	100.0	Bcd	Bcd	Bcd	0	Bcd	Bcd	Bcd	St	Bcd
(Morocco)	10.0	D	0	Bcd	0	0	St	0	St	St
	1.0	0	0	0	0	0	0	0	0	0

BCD = Bactericidal action, no growth after transfer to a new medium.
St = Bacteriostatic action, growth after transfer
D = Temporary delay.
0 = No effect.
Names of antibiotics: 1 = Lincomycin hydrochloride.
2 = Novobiocin sodium.
3 = Streptovaricin C (dalacin C).
4 = Streptozotocin.
5 = Spectinomycin sulfate.
6 = Celesticetin salicylate.
7 = Bluensomycin.
8 = Amicetin.
9 = Streptovaricin complex (dalacin).

strains in sensitivity tests. The table shows that the sensitivity of strain No. 19 is the highest of all four strains. At 100 μgm./ml. concentration with seven antibiotics, a complete bactericidal effect was observed. Strain No. 8 was inactivated completely by only three, and strain No. 12 by one antibiotic. None of the nine antibiotics could inactivate the bacteria of strain No. 9 completely.

Complement-fixation tests with the bacterial antigens obtained from the second and third passages of *M. leprae* on OG medium showed that marked differences existed in the antibody concentrations of the patients' sera. There was no correlation with the bacteriologic finding: e.g., a strong reaction took place with the serum of patient No. 2, who was bacteriologically negative, and of patient No. 13 who was bacteriologically positive. Also the presence of atypical mycobacteria alone or together with *M. leprae* did not correlate with the intensity of the serologic reaction, as shown in Table 6.

Simultaneously with the complement-fixation tests with *M. leprae* antigen, tests were performed with the antigen prepared from the atypical mycobacterium isolated from patient No. 12 (India). Complete inhibition of hemolysis was observed with 0.4 and 0.2 ml. of sera No. 2, 10, 13 and 27. Serum No. 12, the serum of the patient who carried strain No. 12 in his ear lobe (Table 4), reacted with strain No. 12 only when 0.4 ml. were present. At 0.2 ml. no inhibition of hemolysis was observed. The other sera, which were negative or gave weak reactions with *M. leprae* (Nos. 8, 9, 18, 19 and 21), also reacted negatively with antigen from strain No. 12. From the positive sera we have chosen the most active ones and tested them against three different antigens that were available in sufficient quantities. The results are summarized in Table 7. Although from all antigens used half the nonanticomplementary amounts were employed, marked differences in their *in vitro* activity were observed. The most active antigens were No. 10 (India) and No. 13 (Morocco), which gave positive reactions even with 0.02 ml. of serum No. 13 (Morocco) and serum No. 27 (Argentina). The activity of antigen No. 27 (Argentina) was somewhat lower. It gave positive reactions in the

TABLE 6. *Complement fixation with antigen from India-strain No. 10.*

Patient no.	Birth country	Bacillary findings	Intensity of infection at serum quantity (ml.)			
			0.4	0.2	0.1	0.05
2	Libya	Negative	4	4	3	3
8	Yemen	Atypical mycobacteria	0	0	0	0
9	Iran	Atypical mycobacteria	4	0	0	0
10	India	*M. leprae*	4	2	1	0
12	India	*M. leprae* and atypical mycobacteria	1	0	0	0
13	Morocco	*M. leprae*	4	4	4	4
18	Morocco	*M. leprae*	3	0	0	0
19	Morocco	Atypical mycobacteria	0	0	0	0
21	Iraq	*M. leprae*	0	0	0	0
27	Argentina	*M. leprae*	4	4	1	0

4 = No hemolysis.
3 = Traces of hemolysis.
2 = 50% hemolysis.
1 = 75% hemolysis.
0 = Complete hemolysis.

TABLE 7. *Complement fixation of three different patient sera with four different antigens.*

	Quantities of patient sera (ml.)													
	No. 27 (Argentina)					No. 10 (India)				No. 13 (Morocco)				
Antigen	0.4	0.2	0.1	0.05	0.02	0.4	0.2	0.1	0.05	0.4	0.2	0.1	0.05	0.02
No. 27 (Argentina)	4	4	3	1	0	4	0	0	0	4	3	3	1	0
No. 10 (India)	4	4	3	3	3	4	2	1	0	4	4	4	4	4
No. 13 (Morocco)	4	4	3	3	3	4	0	0	0	4	4	3	2	1
No. 29 (Morocco)	4	1	0	0	0	4	1	0	0	4	1	0	0	0

presence of 0.05 ml. of either serum No. 27 or serum No. 13. Antigen No. 29 (Morocco) was a weak one. It reacted with all immune sera only when at least 0.2 ml. of serum were added. The strong sera No. 13 (Morocco) and No. 27 (Argentina) reacted equally with all four antigens. The intensity of the serum reaction was determined by the potency of the antigen only. The weak serum, No. 10 (India), showed some strain specificity. It still reacted with its homologous antigen in the presence of 0.1 ml. serum, and with the two strong antigens, No. 13 (Morocco) and No. 27 (Argentina), only when 0.4 ml. serum were present.

Summarizing these results, we concluded that cross reactions occur between strains of *M. leprae* from widely separate parts of the world and their corresponding patient sera. A definite answer cannot be given as to whether there is complete antigenic identity or whether qualitative or quantitative differences occur in the antigenic structure of the different strains.

DISCUSSION

The experiments described above show that, by the aid of the OG medium with addition of a suitable concentration of malachite-green, it is possible to obtain suspensions of *M. leprae* from the nasal mucosa and the ear lobe skin of leprosy patients, which retain their normal stainability after further transfer on OG medium without malachite-green. The media employed acted also as selective media for other mycobacteria, which were present in three instances in the original specimens alone and in one instance together with *M. leprae*. Although the role of these rapid growing mycobacteria as causative agents has to be determined, we cannot exclude the possibility that some may act as growth-promoters for bacteria-dependent mycobacteria. This possibility has to be investigated by *in vitro* and *in vivo* tests. If there is a symbiosis between saprophytic organisms and bacteria-dependent strains, chemotherapeutic measures should not be concentrated solely on the causative agent, but also on those microorganisms that may act as producers of chelating substances, e.g., mycobactins, or as providers of basic nutrients or higher molecules that may be utilized by the host- or the bacteria-dependent pathogens. The serologic examinations showed that antigenic cross-reactions occur between *M. leprae* and the saprophytic strain No. 12. All the patient sera that gave positive reactions with *M. leprae* reacted also with strain No. 12. This antigenic relationship is only an expression of the fact that a similarity or even identity of chemical groups exists, which is common to pathogenic and saprophytic mycobacteria as well.

SUMMARY

A selective medium has been described, which enabled the isolation and maintenance of uniformly stainable suspensions of *M. leprae* and other mycobacteria. Complement-fixation reactions between antigens prepared from strains of *M. leprae* originating from widely separated countries and the corresponding homologous and heterologous sera from patients, showed that strong cross-reactions occur between patient sera and *M. leprae* antigens originating in different parts of the world. Final conclusions, however, on the antigenic identity of all strains of *M. leprae* could not be drawn.

RESUMEN

Un medio selectivo ha sido descrito que permitió el aislamiento y mantención de suspensiones uniformemente teñidas de *M. leprae* y otras mycobacterias. Las reacciones de fijación del complemento entre los antígenos preparados de cepas de *M. leprae* originarias de países geograficamente alejados y los correspondientes homólogos y heterólogos del suero de los enfermos, mostraron que fuertes reacciones cruzadas ocurren entre el suero del paciente y antígenos de *M. leprae* originario de diferentes partes del mundo. Conclusiones finales, sin embargo, sobre las características antigénicas de todas las cepas de *M. leprae* no pueden establecerse.

RÉSUMÉ

On a décrit ici un milieu sélectif qui permet l'isolement et la persistance de suspensions de *M. leprae* et d'autres mycobactéries colorables de façon uniforme. Les réactions de fixation du complément entre les antigènes préparés à partir de souches de *M. leprae* provenant de pays séparés par des distances considérables, et les sérums homologues et hétérologues correspondants obtenus chez les malades, ont montré que des réactions croisées intenses survenaient entre les sérums du malade et les antigènes de *M. leprae* provenant de différentes parties du monde. Il n'est toutefois pas possible de tirer des conclusions finales quant à l'identité antigénique de toutes les souches de *M. leprae*.

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