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Studies on *Mycobacterium leprae* in Media Enriched by Mycobacterial Extracts [1]

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Many attempts have been made to obtain continuous multiplication of *Mycobacterium leprae in vitro.* Cultures of stromal fibrocytes derived from dorsal root ganglia of human fetuses were employed by Ranadive *et al.* (19) and Bapat *et al.* (1) in the Indian Cancer Research Centre (ICRC). The growth characteristics of the ICRC microorganisms were studied in detail by Bapat *et al.* (2) and their pathogenicity by Ranadive *et al.* (18). Under the same experimental conditions Ganguli (16) succeeded in isolating four slowly growing mycobacterial strains from human lepromatous leprosy. L-cells were employed by Kosaka *et al.* (12), and Yoshie and Sugawara (24) used fibroblasts derived from the skin of a patient with L-type leprosy. Cells derived from human liver, conjunctiva, bone marrow, and intestines were employed by Morris and Nakamura (13), and simian renal cells by Baylet *et al.* (3). Attempts were made by Devignat (4) to cultivate *M. leprae* in symbiosis with other microorganisms. An anaerobic yeast-casein-glycerol medium was employed by Ogata (14) for its cultivation, and Hanks solution with addition of 10 per cent calf serum, by Jadin *et al.* (10). Olitzki and Gershon (16) reported maintenance of the cytopathic activity of *M. leprae* on a modified Eagle's medium supplemented by mycobacterial extracts in four subcultures. They continued their work with the same medium (17) and observed progressing increases of turbidity in it after the inoculation of small amounts of *M. leprae.* Hart and Valentine (9) observed an elongation of *M. lepraemurium,* without multiplication, in a medium that contained casamino acids, asparagine, and albumin as sources of nitrogen, and 7.4 per cent sucrose as a source of energy.

Since the strain isolated by Olitzki and Gershon (16, 17) was not adaptable to other media and grew slowly, further transfers were made on the original medium described, after prolonged incubation periods. In order to improve the growth, nutrients that were recognized as growth-promoting substances for other mycobacteria were added.

Several attempts have been made to initiate *in vitro* growth of host-dependent (i.e., noncultivated) microorganisms, as defined recently by Hanks (7, 8). More than fifty years ago, the chelate-dependent microbe, *M. paratuberculosis,* was cultivated by Twort and Ingram (23) in the presence of mycobacterial extracts. Francis *et al.* (5) selected *M. phlei* as a convenient source of this growth factor. Snow (21) finally established the structure of this factor as

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"mycobactin P" and demonstrated its ability to form very stable complexes with iron and aluminum. Another growth factor, mycobactin T, was isolated by Snow ([22]) from *M. tuberculosis*. The cultivation of microbe-dependent mycobacteria in the presence of fatty acids of mycobacterial origin has been demonstrated by Karlsson ([11]).

In the experiments reported below we employed substances of mycobacterial and of human origin as well. The results obtained in the course of nine transfers within a period of 20 months, are presented.

MATERIALS AND METHODS

Basic medium. The basic medium, a modified Eagle's medium, designated henceforth as BM, contained the following substances, in amounts expressed as gm./liter:

NaCl	6.8
KCl	0.4
$MgSO_4$	0.1
$CaCl_2.2H_20$	0.275
$NaH_2PO_4.H_20$	0.125
Glucose	1.0
L-glutamine	0.3
L-tyrosine	0.75
L-arginine HCl	0.09
L-histidine HCl	0.42
L-isoleucine	0.105
L-leucine	0.105
L-lysine HCl	0.15
L-methionine	0.03
L-phenylalanine	0.067
L-threonine	0.1
L-tryptophane	0.015
L-valine	0.095
L-serine	0.042
Aminoacetic acid	0.03
L-cysteine	0.05

The following vitamins were present in a concentration of 1 mgm./liter: biotin, choline-chloride, folic acid, nicotinamide, calcium pantothenate, pyridoxal, thiamine, riboflavine, and i-inositol. Riboflavin was present in a concentration of 0.1 mgm./liter.

Extracts from atypical mycobacteria as growth-promoting substances. During several transfers, strain No. 2, tested by Olitzki and Gershon ([16, 17]) for its growth-promoting activity, was employed for the extract production. Later another strain, No. 12, was isolated from a patient with lepromatous leprosy, and extracts of it (E12) also were tested for their growth-promoting effect.

The atypical mycobacteria were transferred to fresh Loewenstein's medium and after a suitable period of incubation harvested in 5 ml. of saline/slope. After treatment for three minutes in a MSE-ultrasonic power unit, the bacterial residues were removed by two subsequent filtrations through Sl Seitz filters. The filtrates were autoclaved and tested for sterility on brain-heart infusion broth and Loewenstein's medium. The resulting extracts of strains 2 and 12 are henceforth designated as E2 and E12 respectively. The extracts were added to the BM in a quantity leading to a final concentration of 100 γ protein/ml.

Preparation of foreskin extracts (FSE). Foreskin tissues were cut into small fragments, which were disintegrated in a sterile mortar with the aid of glass powder and addition of 3 ml. of physiologic saline solution pér organ. An additional disintegration of the tissue proteins was effected by a three minute exposure to a homogenizer. Then the suspension was centrifuged for five minutes at 10,000 rpm, and the supernatant sterilized by filtration through a Sl Seitz filter. The protein content was determined by the method of Folin and Ciocalteu. The resulting product is designated hereafter as FSE. The extract was added to BM in a quantity leading to a final concentration of 100 γ protein/ml.

Preparation of foreskin digests (FSD). The tissues were cut and disintegrated as for FSE. The pH was adjusted to 4.0 with lN HCl and the product was treated with pepsin for four hours. The suspension was centrifuged, the pH was readjusted to 7.0 with concentrated NaOH solution, and the suspension was then filtered through the Seitz filter. The digest was used in a quantity leading to a concentration of 100 γ protein/ml.

Approximate determination of the microorganism in the culture fluid. *M. leprae* produced large flakes in the enriched medium, which sedimented rapidly to the bottom of the test tubes. Therefore, before observing the turbidity, vigorous shaking of the test tube was necessary. Even then the bacteria settled quickly to the bottom. Since it was impossible to produce a homogeneous suspension, we finally abstained from photometric measurements. Furthermore, the appearance of a homogeneous turbidity in a test tube always indicated a nonmycobacterial contamination. Microscopic examination of the sedimented flakes stained by the Ziehl-Neelsen technic always confirmed the macroscopic observation, i.e., growth of *M. leprae* at the bottom of the test tube, leaving a clear supernatant culture fluid. Even when 0.05 per cent Tween 80 was added, a nonhomogeneous suspension was obtained.

We finally employed an approximate estimation in counting the microorganisms. After short vigorous shaking of the culture 0.05 ml. was taken with the aid of a standard pipette and disposed on a circle, marked on the slide, 5 mm. in diameter. By counting a sufficiently large number of fields, the following degrees of bacterial density were determined: ±, one single bacterium in more than 100 fields; 1+, one single bacterium or a group once in less than 100 fields; 2+, an average of no more than 10 bacteria per field; 3+, an average of 11-30 bacteria per field, and 4+, masses of bacteria per field and globi.

The bacterial structure. We paid attention also to the structure of the cultivated bacteria. According to Rees and Valentine [20], degenerated leprosy bacilli appear under the microscope as irregularly stained, while viable forms show uniform staining. Infectious material taken from a treated patient, in which degenerated bacteria were prevalent, served as a control for the cultured bacteria.

Experimental procedure. In order to examine the effect of varying incubation periods on the viability of the bacteria, successive transfers were made at about two month intervals, and simultaneously from the 2nd to the 6th *in vitro* culture after varying incubation periods up to eight months. After these prolonged incubation periods, bacteria were transferred to further subcultures in order to examine the effect of the prolonged incubation on the viability of the microorganisms.

Origin of the mycobacterial strain. All cultures were derived from the line cultured since 2 March 1965 by Olitzki and Gershon [16, 17]. On that date the infected material was inoculated in BM enriched by E2. It was transferred on 18 April 1965 to the same medium for further passages.

RESULTS

First experiment. Subcultures from the 2nd *in vitro* culture of 18 April 1965 were kept in the incubator at 37°C, until 24 December 1965. Then quantities of 0.1 and 1.0 ml. were transferred to fresh BM enriched by E2. An increasing turbidity was observed and microscopic examination, performed on 22 February 1966, revealed masses of acid-fast bacteria.

This experiment proved that after eight months of incubation at 37°C, the viability of *M. leprae* was still maintained. The microscopic picture revealed homogeneously stained bacteria. No bipolar or granular forms were present.

Second experiment. On 26 May 1965 specimens of the 2nd *in vitro* cultures from the 18 April 1965 culture were diluted up to 10^{-7}, and 1.0 ml. of each dilution was transferred to BM enriched immediately by E2. To another group of cultures E2 was added about seven months later, on 20 December 1965. All these 3rd *in vitro* cultures were incubated from 26 May until 24 December 1965. On that date transfers from the 3rd to the 4th *in vitro* cultures were made. The new media were enriched by E2. The results are presented in Table 1.

Table 1 shows that the viability of the bacteria on the medium enriched immediately by E2 and also on the later enriched media, was maintained for seven months at 37°C. Even the inocula diluted up to 10^{-6} still contained viable bacteria, which, after a further passage on enriched media, were able to produce an abundant growth within two months.

The whole procedure may be summarized as follows:

Infected material
↓
on 2 March 1965
↓
1st *in vitro* culture
↓
on 18 April 1965
↓
2nd *in vitro* culture —— on 24 December 1965 ——→ **3rd *in vitro* culture**
↓
on 26 May 1965
↓
3rd *in vitro* culture —— on 24 December 1965 ——→ **4th *in vitro* culture**
↓
on 1 July 1965
↓
4th *in vitro* culture —— on 20 December 1965 ——→ **5th *in vitro* culture**
↓
on 1 September 1965
↓
5th *in vitro* culture —— on 20 December 1965 ——→ **6th *in vitro* culture**
↓
on 8 November 1965
↓
6th *in vitro* culture —— on 10 February 1966 ——→ **7th *in vitro* culture**
↓
on 27 December 1965
↓
7th *in vitro* culture
↓
on 10 & 12 February 1966
↓
8th *in vitro* culture
↓
on 20 May 1966
↓
9th *in vitro* culture

The delayed addition of E2 on 20 December 1965 to the 3rd *in vitro* culture did not limit the capability of the bacteria to grow after the 4th *in vitro* culture in the presence of E2. Under both conditions the inoculum taken from the 3rd *in vitro* culture, which had received an inoculum of 10^{-6} ml. previously, grew in the 4th *in vitro* culture. There was a difference, however, in the cultures that had received an inoculum of 10^{-7} ml. Those that had simultaneously received an addition of E2 showed an abundant growth in the 3rd and 4th *in vitro* cultures. Those that had received E2 after a delay of seven months stopped growing in the 3rd passage, and after the transfer of 1.0 ml. to a new enriched medium no growth occurred.

Third experiment. On 1 July 1965 bacteria were transferred from the 3rd to the 4th *in vitro* culture. About six months later another transfer to the 5th *in vitro* culture was made. Although the cultures were incubated for almost six months, turbidity appeared and masses of bacteria were detected on 22 February 1965, as shown in Table 2.

Fourth experiment. On 1 September 1965 inocula were transferred from the 4th to the 5th *in vitro* culture. To one group of the cultures human plasma was added to a concentration of 10 per cent. E2 was added to both groups on 20 December 1965. On the same day inocula of 1.0 and 0.1 ml. from each culture tube were transferred to the 6th *in vitro* culture. The results of this experiment are summarized in Table 3.

Table 3 shows that growth did not appear within two months of incubation un-

TABLE 1. *Growth of mycobacteria incubated from 26 May 1965 to 22 February 1966, of subcultures retained from 24 December 1965 to 22 February 1966, and effect of delayed addition of E2 on their growth.*[a]

Transfer from 2nd to 3rd *in vitro* culture on 26 May 1965				Transfer from 3rd to 4th *in vitro* culture on 24 Dec. 1965		
E2 added	Inocula in ml.	Growth observed on 20 Dec. '65	Growth observed on 22 Feb. '66	E2 added	Inocula in ml.	Growth observed on 22 Feb. '66
26 May '65	10^{-6}	++	+++	24 Dec. '65	1.0	+++
	10^{-7}	++	++		1.0	+++
20 Dec. '65	10^{-3}	+	+++	24 Dec. '65	1.0	+++
					0.1	+++
	10^{-4}	+	+++		1.0	+++
					0.1	++
	10^{-5}	—	+++		1.0	+++
					0.1	++
	10^{-6}	—	+++		1.0	+++
					0.1	+++
	10^{-7}	—	—		1.0	—
					0.1	—

[a]The following abbreviations are used in this table and in Tables 2 and 3.
E2 = Mycobacterial extract prepared from strain No. 2.
— = No bacteria found microscopically.
1+ = One bacterium or a group once in less than 100 fields.
2+ = An average of no more than 10 bacteria/field.
3+ = An average of 11-30 bacteria/field.
4+ = Masses of bacteria/field and globi.

TABLE 2. *Growth of cultures incubated from 1 July 1965 to 24 December 1966.*

Transfer from 3rd to 4th *in vitro* culture on 1 July 1965			Transfer from 4th to 5th *in vitro* culture on 20 Dec. 1965		
E2 added	Inocula in ml.	Growth observed on 22 Feb. 1966	E2 added	Inocula in ml.	Growth observed on 22 Feb. 1966
1 July '65	10^{-3}	++	20 Dec. '65	1.0	+++
				0.1	+++
	10^{-4}	++		1.0	++
				0.1	++
	10^{-6}	+		1.0	+++
	10^{-7}	—		1.0	—

less E2 was added. Human plasma did not promote growth without the presence of E2. When E2 was added, however, growth became more abundant. On 20 December 1965, another transfer was made from the 5th to the 6th *in vitro* culture. Human plasma was not added, but the presence of E2 alone was sufficient to promote marked growth.

Fifth experiment. On 8 November 1965 diluted inocula were transferred from the 5th to the 6th *in vitro* culture. In order to enhance the growth, FSD, for the first time, was added. On 27 December 1965 growth was observed in cultures in which the inoculum was diluted up to 10^{-4}. On the same day another transfer was made from the 6th to the 7th *in vitro* culture. E2 and

FSD were added again. On 22 February 1966 growth appeared in tubes of the 6th *in vitro* culture, in which the inoculum was diluted up to 10^{-4}. Growth occurred in the 7th *in vitro* culture up to an inoculum dilution of 10^{-3}.

Sixth experiment. On 10 February 1966 other transfers from the 6th to the 7th *in vitro* culture were made; the 6th as well as the 7th *in vitro* culture contained both E2 and FSE. Since the bacterial growth in the higher dilution of the inocula (10^{-5} to 10^{-7}) was no more marked, the media from the 7th passage were enriched with E2, FSE, 0.5 per cent glycerol (henceforth designated as G) and 0.05 per cent Tween

TABLE 3. *Growth of bacteria incubated from 1 September 1965 to 22 February 1966 and of subcultures retained from 24 December 1965 to 22 February 1966.*

Transfer from 4th to 5th *in vitro* culture on 1 Sept. 1965				Transfer from 5th to 6th *in vitro* culture on 20 Dec. 1965		
Substances added	Inocula in ml.	Growth observed on 20 Dec. '65	Growth observed on 22 Feb. '66	Substances added	Inocula in ml.	Growth observed on 22 Feb. '66
E2 on 20 Dec. '65	10^{-5}	−	+	E2 on 20 Dec. '65	1.0	++
					0.1	++
	10^{-6}	−	−		1.0	++
					0.1	+
	10^{-7}	−	−		1.0	−
					0.1	−
Human plasma to concentration of 10% on 1 Sept. and E2 on 20 Dec. '65	10^{-4}	−	+++	E2 on 20 Dec. '65	1.0	+++
					0.1	+++
	10^{-5}	−	+++		1.0	++
					0.1	++
	10^{-6}	−	−		1.0	+++
					0.1	+
	10^{-7}	−	−		1.0	−

TABLE 4. *Microscopic observations on mycobacteria grown in the 7th* in vitro *culture enriched by different substances after incubation from 10 February to 10 August 1966.*[a]

Medium	Substances present in medium					Growth	
	BM	E2	FSE	G	T80	Intensity	Details
1	+	+	−	−	−	+++	Groups of bacteria in each field
2	+	+	−	+	−	+++	Groups attached to insoluble particles, in each field
3	+	+	−	+	+	++++	Single bacteria and globi containing masses of bacteria
4	+	+	+	−	−	++	Small groups in each field
5	+	+	+	−	+	+	A few groups in several fields
6	+	+	+	+	+	+	A few groups in several fields

[a] The following abbreviations are used in this table and in Table 5. Growth intensities from 0 to 4+ recorded as in Tables 1-3.

EM = Eagle's medium.
E2 = Mycobacterial extract prepared from strain No. 2.
FSE = Foreskin extract.
G = 0.5 per cent glycerol.
T80 = 0.05 per cent Tween 80.
+ = Substance present.
− = Substance not added.

80 (T80) in different combinations. Microscopic observations made on the different cultures are summarized in Table 4.

Seventh experiment. Another passage was made on 10 February 1966, and on 23 February 1966 from the 7th to the 8th *in vitro* cultures. In one group the source of the inoculum was a culture enriched with E2 only, and in another a culture enriched with E2 and FSD. The results of this experiment are summarized in Table 5.

Table 5 shows that in the 8th *in vitro* culture all these cultures that originated from the parent culture, which contained E2 and FSD, showed more abundant growth than those originating from the culture enriched only by E2. In one culture enriched by E2 and G no growth occurred, while in another with the same enrichment, but originating from a parent culture enriched with E2 and FSD, an abundant growth occurred.

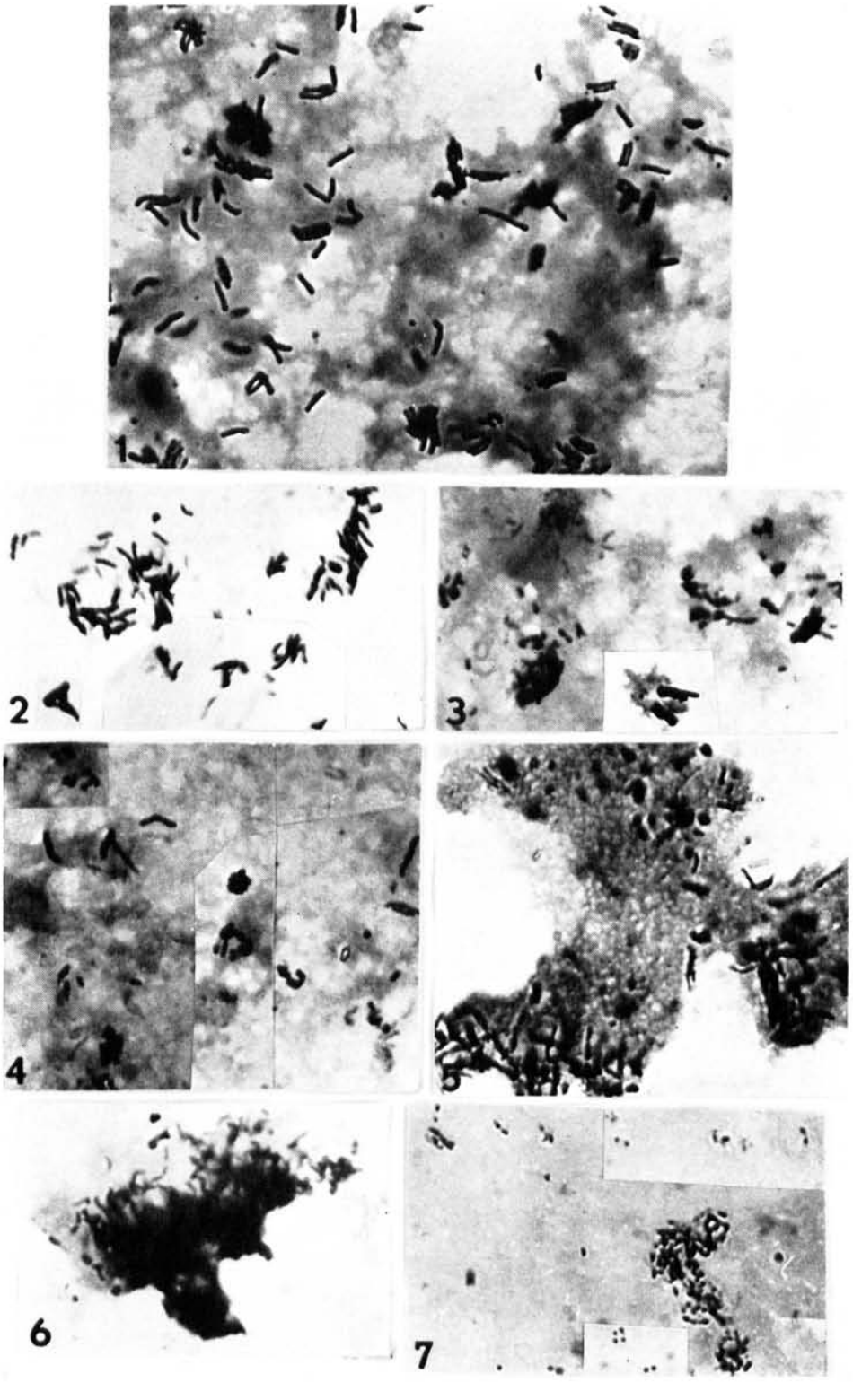
In the meantime, an atypical mycobacterium was isolated from a patient with lepromatous leprosy. This strain grew on all conventional media employed for the growth of mycobacteria. Its extract also promoted the growth of *M. leprae*. This extract, designated E12, was added to all media of the 9th *in vitro* culture. In view of experience in the 8th *in vitro* culture we also added, besides FSE, an equal quantity of FSD.

In addition we examined the following alcohols for their eventual growth-promoting action: ethanol, methanol, glycerol, erythritol, sorbitol, and mannitol. Other substances examined were glycerophosphate and bovine albumin.

TABLE 5. *Microscopic observations on mycobacteria grown in the 8th* in vitro *culture enriched by different substances after incubation from 10 and 22 February 1966 respectively to 10 August 1966.*

Original culture enriched by	Substances present in medium					Growth	
	BM	E2	FSE	G	T80	Intensity	Details
E2 (transfer on 3 Feb. '66)	+	+	−	−	−	++	Small groups attached to insoluble particles
	+	+	−	+	−	0	No bacteria
	+	+	−	+	+	+	A few single bacteria
	+	+	+	−	−	+	Small groups of bacteria
	+	+	+	+	−	+	A few single bacteria
	+	+	+	+	+	+++	In each field single bacteria and globi
E2 + FSD (transfer on 10 Feb. '66)	+	+	−	−	−	+	Bacteria in small groups
	+	+	−	+	−	++++	Masses of bacteria and globi
	+	+	−	+	+	++	Bacteria concentrated in globi
	+	+	+	−	−	++	Groups and globi in each field
	+	+	+	+	−	++++	Masses, single and globi
	+	+	+	+	+	++++	Masses of bacteria

FIG. 1. *M. leprae* from the 3rd passage. FIG. 2. Groups of mycobacteria from the 4th passage. FIG. 3. Groups of bacteria from the 6th passage. FIG. 4. Groups of bacteria from the 7th passage. FIG. 5. A group of bacteria from the 8th passage attached to an insoluble particle (Table 5). FIG. 6. A microcolony of acid-fast bacteria from the 8th passage. FIG. 7. Control: *M. leprae* from a case of lepromatous leprosy. The majority of the microorganisms show bipolar staining. (*Note:* Ziehl-Neelsen staining all slides. Magnification x 1,350.)


1
2
3
4
5
6
7

The observations of the 9th *in vitro* culture, incubated from 20 May to 30 August 1966 did not reveal any growth-promoting or inhibiting effect of these substances.

The bacterial structure. Figures 1, 2, 3, 4, 5 and 6 show that in all passages the morphologic structure and uniform stainability of the mycobacteria were retained. Figure 7 shows the microorganisms that served as controls, after staining with the same standard Ziehl-Neelsen acid-fast method. They were taken from a case of lepromatous leprosy under prolonged treatment. The differences in the morphology between the bipolar control bacteria and the uniformly stained cultivated bacteria are evident.

DISCUSSION

The results of the experiments described above, may be interpreted differently. If we assume that on the enriched medium no multiplication took place, then we have to consider the following facts. For each subculture no more than 1.0 ml. of the parent culture was transferred; in several experiments the amount was 0.1 ml. or less. In the course of eight subcultures this procedure was repeated eight times and, as a consequence, the original infective material was subjected eight times to a tenfold dilution. This means that the 8th *in vitro* culture contained 10^{-8} microorganisms of the initial inoculum. Actually, in many culture tubes of the 8th *in vitro* culture, there were masses of bacteria, as shown in Table 3 and in Figures 5 and 6. If the bacteria did not multiply, then the appearance of microcolonies in the 8th *in vitro* culture, as shown in Figure 6, might be explained as follows: either these colonies preexisted in the infective material and had been transferred eight times, or aggregates of bacteria were produced by an agglutinating factor present in the nutrient medium. These explanations, however, seem insufficient to explain the presence of such bacterial masses in the 8th *in vitro* culture. If we suppose that in the original inoculum 10^{12} bacteria/ml. were present, then, without multiplication, the bacterial population in the 8th subculture should be no greater than 10^4/ml. Such a number is too small to give microscopic pictures such as those shown in Figures 5 and 6; in the slides corresponding to these figures masses of single bacteria and clumped bacteria were found in almost every microscopic field. We have to assume, therefore, that in the course of several subcultures some multiplication has taken place. Additional experiments will show whether the bacterial growth will continue in additional subcultures or not. The possibility exists also that the cultured bacteria were not identical with *M. leprae*, but merely saprophytic mycobacteria. Since we did not succeed in cultivating them on the conventional media employed for cultivation of mycobacteria, there is no evidence that we were dealing with saprophytes.

Several biologic activities of this strain were demonstrated from the original inoculum up to the 4th passage. Olitzki and Gershon [16] reported its cytopathic activity on murine monocytes, and Olitzki [15] described the production of necrotizing reactions in leprosy patients, which were different from the allergic reaction of the lepromatous type or from the Mitsuda reaction, and seemed to present rather an analogy to the Koch phenomenon. This reaction was produced by viable bacteria only. Bacteria killed by heating at 100°C were unable to evoke it. There is a possibility also that only specific strains of *M. leprae*, or selected mutants, were able to grow *in vitro* on the medium described above, while the majority of the strains of this species are strictly host-dependent. In order to examine these possibilities, attempts were made to isolate and cultivate additional strains from other sources. The results of these experiments will be reported in another publication.

SUMMARY

Mycobacterium leprae was subcultured eight times on a modified Eagle's medium enriched by an extract derived from saprophytic mycobacteria and by foreskin extracts or digests. Eighteen months after the first inoculation of the infective ma-

terial numerous uniformly stained bacteria were still present in the 8th *in vitro* subculture. It is not clear whether the fortifying substances acted as growth factors, chelating agents, or maintenance factors. But from observations made on the more recent subcultures *in vitro*, where the host substances were highly diluted or completely lacking, it became clear that the *in vitro* growth and/or maintenance of *M. leprae* do not depend upon a single factor. Several chemical factors of bacterial and/or host origin, together with physical conditions of the cultural environment, seem to contribute to the maintenance and eventual growth of this microorganism *in vitro*. Each one of these factors should be defined qualitatively and its action determined quantitatively.

Furthermore, it is not clear whether or not strains of *M. leprae* originating from various geographic areas react equally under the same *in vitro* conditions. It is possible that strains of other origin may react in another way than the specific strain that was employed in the experiments described above. For this reason additional investigations have been performed with several strains originating from remote geographic zones. The results obtained with these strains will be published.

RESUMEN

El *Mycobacterium leprae* fué subcultivado mediante ocho fases en un medio modificado de Eagle enriquecido por un extracto derivado de mycobacterias sapróphitas y por un macerado o extracto de piel de prepucio. Diez y ocho meses después de las primeras inoculaciones del material infeccioso numerosas bacterias uniformemente teñidas todavía estaban presentes en el 8° subcultivo *in vitro*. No es claro si las substancias fortificantes actuaron como factores de crecimiento o como agentes relacionadores, o de mantenimiento. Pero de las observaciones hechas de los subcultivos *in vitro* mas recientes, donde les substancias propias del huésped estaban altamente diluídas o totalmente ausentes, fué claro que el crecimiento *in vitro* y/o el mantenimiento de *M. leprae* no depende de un solo factor. Varios factores químicos del bacteria y/o del huésped de orígen, juntos con condiciones físicas del ambiente de cultivo, parecen contribuir a las mantención y crecimiento eventual de este microorganismo *in vitro*. Cada uno de estos factores debe ser definido cualitativamente y sus acciones deben ser determinadas cuantitativamente.

Mas todavía, no es claro si cepas de *M. leprae* originarias de varias áreas geográficas reaccionan igualmente en las mismas condiciones *in vitro*. Es posible que cepas de otro orígen puedan reaccionar de otra manera que la cepa específica que fué empleada en los experimentos descritos anteriormente. Por esta razón investigaciones adicionales se han hecho con varias cepas originarias de zonas geográficas remotas. Los resultados obtenidos con estas cepas serán publicados.

RÉSUMÉ

Mycobacterium leprae a été cultivé et transféré huit fois de culture à culture sur un milieu modifié de Eagle enrichi par un extrait dérivé de mycobacteries saprophytes et par des extraits de prépuce ou par des préparations digérées du même matériel. Dix-huit mois après la première inoculation du matériel infectieux, de nombreuses bactéries colorées de manière uniforme étaient encore présentes dans la culture *in vitro* obtenue à la suite du huitième transfert. Ce qui reste peu clair, c'est si les substances fortifiantes ont agi comme facteur de croissance, comme agent de chelation, ou comme facteurs de soutien. Cependant, des observations faites sur les cultures *in vitro* les plus récentes, dans lesquelles les substances de l'hôte étaient fortement diluées ou bien manquaient complètement, il est devenu évident que la croissance *in vitro*, de même que la persistance de *M. leprae*, ne dépendent pas d'un facteur unique. Plusieurs facteurs chimiques qui proviennent, soit de la bactérie, soit de l'hôte, ou bien des deux à la fois, de même que les conditions physiques du milieu de culture, semblent contribuer à la persistance et à la croissance eventuelle de ce microorganisme *in vitro*. Chacun de ces facteurs devrait être défini qualitativement et son action déterminée de façon quantitative.

De plus, on ignore si les souches de *M. leprae* provenant de diverses régions géographiques réagissent de la même manière dans des conditions *in vitro* identiques. Il est possible que les souches d'autre origine puissent réagir

d'une autre façon que la souche spécifique qui fut employée dans les expériences décrites ci-dessus. Pour cette raison, des recherches complémentaires ont été menées avec diverses souches provenant de régions géographiques reculées. Les résultats obtenus avec ces souches seront publiés.

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