AN ACID-FAST MICROORGANISM CULTIVATED FROM LEPROUS MATERIAL. BACTERIOLOGICAL AND SEROLOGICAL OBSERVATIONS

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INTRODUCTION

In our attempts to cultivate the leprosy bacilli *in vitro*, we have employed both Dubos' medium, which was devised for the rapid culture of mycobacteria, and the larva of the wax moth, *Galleria mellonella*, an insect which is very receptive to tuberculous infection. In the course of this work we have cultivated from a leprous subject a slow-growing acid-fast microorganism. Some of the properties of this organism are considered in this article; a more detailed presentation of bibliographical and experimental data will be published elsewhere. (11)

As is well known, it is still a matter of discussion whether or not the leprosy bacillus can be cultivated. If it is possible to cultivate it, certainly the culture is very difficult to obtain.

Since Hansen, after observing the leprosy bacillus in the lesions, made the first unsuccessful attempts to cultivate it outside the body many workers have claimed that they have succeeded while others even now are doubtful that it has been accomplished. Many substances of animal, vegetable and microbial origin have been included in culture media to induce *in vitro* growth of the Hansen bacillus, and some workers have used living bacteria, or protozoa, or even animal tissue cells (tissue cultures) in order to obtain a kind of symbiosis.

Cultures which have been obtained are of many different kinds. Some appeared to be very similar to those of leprosy, while others were completely different. Among the acid-fast bacteria cultivated, some were readily growing, probably saprophytes, while others were slow-growing and often very difficult to carry on beyond the first isolation. The latter are somewhat similar to leprosy bacilli, although their identity cannot be surely demonstrated. Recently Penso and Ortali (9) have isolated bacteriophages active against different varieties of acid-fast organisms, and it is hoped that identification of germs culti-

vated from leprosy may be accomplished by this method. Leprosy has not been constantly reproduced in laboratory animals or in man by inoculating cultures grown from leprous material. Even experimental infection of man by inoculation of leprous material has almost always been unsuccessful, although accidental inoculation has been reported occasionally.[1]

Other bacteria cultivated from leprosy material include various forms, often nonacid-fast: coccoid, diphtheroid, streptothricial and others. Some organisms cultivated are constant in their morphological properties while others are morphologically variable, so that some students believe in the existence of "development cycles" with rare acid-fast phases. Some of the germs which have been cultivated, especially by earlier students of the subject, are probably mere contaminants.

With regard to serological reactions in leprosy, the most specific ones are the Rubino reaction (agglutination of formalin-treated sheep's red corpuscles) and complement-fixation reactions with acid-fast bacillary antigens (organisms cultivated from leprous material and others). Complement-fixation reactions with actual leproma material, though sensitive, are less specific. Other serological reactions used in other diseases, especially that of Wassermann, tend to give false positive results in leprosy. As is well known, leprosy sera are often "polyreactive," giving positive responses to various tests.

EXPERIMENTAL

For our bacteriological work we have had at our disposal a total of 17 persons with leprosy,[2] the most notable among them being a woman newly attacked by the disease who had not yet been treated and who had plenty of bacilli in her nodules. From these patients the cultivation of the Hansen bacillus was attempted by means of techniques not often used.

As said, in this work we employed Dubos' medium and larvae of *G. mellonella*. Mycobacteria develop rapidly and homogeneously in Dubos' medium (3, 4, 5), which has been used recently by Chaussinand (2) in an attempt to culture the leprosy bacillus,

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[1] The most recent and convincing report of accidental inoculation is that of Porritt and Olsen (10).

[2] In total, we have studied 34 cases of leprosy, from the following sources: 1 here in Florence, but from southern Italy; 1 from Parma, in northern Italy, autochthonous; 1 in Bologna, a missionary from China; 5 autochthonous ones in Lucca; 10 in the leprosarium in Bari; and 16 in the leprosarium in Genoa.

the results of which were negative. The larval form of *G. mellonella*, used first in bacteriological studies by Metalnikov (6, 7), allows rapid multiplication of bacteria inoculated into the body cavity (8).

Samples of the leprous nodules and blood, taken aseptically, were often homogenized with 4 per cent NaOH or 10 per cent H_2SO_4, or used without homogenization. Nasal mucous, also used, was always homogenized.

Materials from 10 nodules were inoculated into Dubos' medium, and from 12 nodules into 33 Galleria larvae (0.025 to 0.012 ml. of the suspension in saline). From 4 of the latter, material was subinoculated into 8 others, and from 3 of them into tubes of Dubos' medium. From all the samples of leprous material and all the Galleria larvae, tubes of Petragnani's medium and of glycerol potato were inoculated. Nasal mucus from 2 patients was inoculated into 4 larvae, and 1 sample of blood of a patient recently attacked by acute nodular leprosy into tubes of Dubos' medium. On microscopic examination most of the samples appeared full of acid-fast bacilli.

The cultures and the Galleria larvae were incubated aerobically at 37°C. for varying periods of time. If the larvae did not die soon after the inoculation, they were killed and inoculated into Dubos' and Petragnani's media, generally after a week. If bacilli were found in the dead larvae, such inoculations into other ones were made. Cultures were incubated for 6 months before they were considered sterile.

All of the experiments were negative, except the following: A skin leproma from a nodular case was inoculated aseptically into 6 larvae, and into Petragnani's and Dubos' media and glycerol potato. Two days later material was transferred from one Galleria onto those three media. All of these cultures remained sterile, except one. In a tube of Petragnani's medium one colony was observed three months after inoculation from the larva. The colony consisted of acid-fast gram-positive bacteria, aerobic, nonencapsulated, nonspore-forming and nonmotile.

Subcultures develop better at 37°C. than at 25°C.; they do not develop at all at 15°C. Growth is observed in from 15 to 25 days on Petragnani's medium, glycerol potato, glycerol agar (smooth, yellow), and glycerol broth (uniform turbidity, no surface pellicle). Development is rapid on Dubos' medium, occurring in two days. No development is observed in Loeffler's serum, nor on blood agar. Gelatin is not liquefied; indol and H_2S are not produced; liquid media are alkalinized; nitrates and neutral red are reduced; the Voges-Proskauer reaction is negative. The yellow, nondiffusing pigment is much more abundant after incubation at 37° than at 25°C. It is soluble in some fat solvents.

Glycerol is necessary for development, and some protein derivatives are useful. Certain accessory growth factors, such as

TABLE 1.—*Complement-fixation reactions with sera from leprosy and control cases, at different dilutions.*

Antigen	Leprosy patients							Control patients						
	No. of cases	No. negative	No. positive					No. of cases	No. negative	No. positive				
			1/20	1/40	1/80	1/160	1/320			1/20	1/40	1/80	1/160	1/320
Methylic extract	34	6	4	5	4	6	9	53	49	4	0	0	0	0
Whole bacillary	34	11	7	11	2	3	0	63	58	5	0	0	0	0
Tuberculosis	34	29	2	2	1	0	0	62	61	1	0	0	0	0

TABLE 2.—*Type distribution of leprosy cases giving positive complement-fixation in 1/40 dilution or higher.*[a]

Antigen	Nodular (26 cases)		Anesthetic (8 cases)		Total (34 cases)	
	Positive	Per cent	Positive	Per cent	Positive	Per cent
Methylic extract	21	81	4	50	25	76
Whole bacillary	14	54	2	25	16	47
Tuberculosis	3	12	0	0	3	9

[a] Of the controls, none gave a positive reaction in 1/40 dilutions, as shown in Table 1.

pantothenic, para-aminobenzoic and nicotinic acids and vitamin K, failed to affect the development.

The microorganism is not pathogenic for rabbits, guinea-pigs, rats or mice when inoculated parenterally (2 mgm., dry weight).

This culture was used to prepare both whole bacillary and methylic extract (Boquet and Nègre) antigens for a study of the complement-fixation reaction with sera of leprous persons and of controls with other diseases (abdominal, cutaneous and pulmonary tuberculosis, syphilis, and others). Besides these, a mixed tuberculosis antigen (a suspension of human, bovine and avian bacilli: "anatubercolina intergrale," Petragnani) was used.[3]

For the reactions, 0.5 ml. doses were used (total volume 2.5ml.). The sera were inactivated for 20 minutes at 55°C. The complement was fresh serum of 5 guinea-pigs in a 1/20 dilution. Sheep red corpuscles were used in a 5 per cent suspension. The amboceptor was a rabbit antisheep serum. All dilutions were made with saline. After fixation of the complement (90 minutes at 37°C. in water bath), the hemolytic system was added (red corpuscles sensitized with 3 hemolytic doses) and the incubation was continued for 1 hour more.

Sera giving positive reactions, even partial, in a dilution of at least 1/40 were considered positive, because even in the case of normal sera reactions can occur—though they seldom do—in a 1/20 dilution. No serum was anti-complementary in a 1/40 dilution.

The results of these reactions, shown in Tables 1 and 2, seem to be comparable to those obtained with other acid-fast antigens as reported by other authors. The difference between the percentage of positive leprosy sera with the internal or the methylic antigen and that with the control sera is statistically significant in 1/20 sera dilutions.[4] With 1/40 sera all of the controls were negative while most of the leprous sera were still positive.

The tuberculosis antigen is far less sensitive, though it gives more frequent positive reactions among leprosy cases than among controls.

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[3] Flocculation reactions with the methylic extract as the antigen, and agglutination reactions with a suspension of bacilli, were also made, with poor results.

[4] The significance was calculated from the formula:

$$\text{Percentage difference (in decimals)} > 2 \cdot \sqrt{\frac{p'\,q'}{n'} + \frac{p''\,q''}{n''}}$$

p′ and p″ represent the percentages (in decimals) of each observation series;
q′ and q″ are the complementary numbers of p′ and p″;
n′ and n″ are the numbers of observations in each series.

COMMENT

It is of course impossible to say whether the bacilli which we cultivated are the Hansen bacilli or saprophytic contaminants, either from the patient's skin or from the Galleria larva. However, the following facts seem to support the hypothesis that they are neither saprophytic contaminants nor tubercle bacilli.

1. A single colony was isolated from material full of acid-fast bacteria. If those which developed were paratubercle bacilli they would probably have produced a luxuriant culture.

2. The cultivation of this microorganism is more difficult than that of most paratubercle bacilli.

3. They are not lysed by bacteriophages[5] active against the following bacilli: *Myco.* Pellegrino, *phlei* Rabinowitsch, *lacticola, smegmatis.*

4. They hardly ever multiply except at 37°C.

5. Repeated attempts at obtaining cultures from the patient's skin on media suitable for mycobacteria were always unsuccessful.

6. The results of the serological reactions suggest that the bacteria are more like the Hansen bacillus than the tubercule bacilli.

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