Cord-Forming Property, Lethality and Pathogenicity of Mycobacteria*

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Introduction

The genus Mycobacterium include a great number of non-pathogenic organisms as well as some pathogenic species which cause tuberculosis in mammals, birds and cold-blooded animals.

The differentiation between pathogenic strains and non-pathogenic ones is based on the lethal properties of the bacilli for experimental animals, particularly the guinea pig. A strain is not considered pathogenic when it does not kill the animal, even when it causes considerable morbid lesions. This simple criterion of pathogenicity makes it possible to differentiate the lethal strains from non-pathogenic or saprophytic Mycobacteria but it does not enable to recognize the non-lethal strains of reduced or intermediate pathogenicity. It was accepted by inference, that these latter strains are not of importance in human pathology. Interest in these strains was aroused recently when it was found that the cultures of acid-fast bacilli from patients under streptomycin or isoniazid therapy are often not lethal to guinea pigs.*

The discovery that these strains although not lethal to guinea pigs are lethal to mice (Karlson and Ikemi, 1952), that the BCG strain, which was thoroughly studied and recognized as not lethal to animals and humans, has killed two men (Meyer and Jensen, 1954; Oeding and co-workers, 1954) further complicated the pathogenicity problem of Mycobacteria. The amount of lesions in animals cannot differentiate the strains of reduced pathogenicity. Other suggested procedures, such as the inoculation of the chorioallantoic membrane of the developing chick embryo, have also failed to differentiate Mycobacteria of reduced pathogenicity (Fite and Olson, 1944).

In vitro methods would be of considerable advantage over animal inoculation in pathogenicity studies. They would save time and money and might eventually demonstrate the pathogenicity of Mycobacteria not revealed by animal inoculations. Middlebrook, Dubos and Pierce, 1947, recognized the “cord formation” in Mycobacteria as an indicator of pathogenicity. These findings were confirmed by Roth, 1949, Bloch, 1950, and others. Doubts about the significance of “cord formation” as an indicator of pathogenicity were aroused when this kind of formation was found in seemingly non-lethal or even saprophytic strains of Mycobacteria, (Richmond and Cummings, 1950) and when photographs of BCG cords were published by Kölbel, 1951, Engbaek, 1952. These findings forced Dubos, 1950, to pose the question of how to reconcile the presence of cords and the absence of pathogenicity in Mycobacteria.

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The above considerations led us to study cord formation as a means of differentiating degrees of pathogenicity of Mycobacteria.

**Method**

A previous study by one of us (E. D.) has shown that a liquid medium of the following composition stimulates cord formation:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄</td>
<td>1.0 gram</td>
</tr>
<tr>
<td>Na₂HPO₄·12H₂O</td>
<td>5.0 gram</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.2 gram</td>
</tr>
<tr>
<td>Asparagine</td>
<td>2.0 gram</td>
</tr>
<tr>
<td>Peptone, Bacto</td>
<td>5.0 gram</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>1.0 gram</td>
</tr>
<tr>
<td>Ferris ammon. citrate</td>
<td>0.02 gram</td>
</tr>
<tr>
<td>Dist. water</td>
<td>1000.0 gram</td>
</tr>
</tbody>
</table>

The basal medium is distributed in flasks and sterilized in an autoclave.

Blood from the carotid artery of a freshly killed ox is aseptically collected in a sterile, paraffinized 1 liter Erlenmeyer flask. (Dissolve 1-2 grams of hard paraffin in ether, rinse the flask with the solution, discard the rest. The paraffinized flask may be used some days later, when the ether has evaporated). On the second and third day the serum is aseptically collected from the clotted blood, centrifuged, divided into 50 ml. lots in 250 ml. flasks, heated for one hour in a water bath at 56° C. After cooling, 15 ml. of sterile glycerol is added to each 50 ml. of the heated serum. After a week or two a sterility test of the mixture is made. Conserved in an ice-box, it is good for months.

To make up the culture, 10 ml. of glycerinated serum are added to 100 ml. of basal medium. The medium is aseptically distributed into big tubes (3 x 15 cm.), 6-8 ml. per tube. The inoculated tubes are incubated at 37° C. for two weeks, then 0.2-0.3 ml. of the sediment is taken out with an ordinary pipette, spread over a slide, dried and stained according to the Ziehl-Neelsen technique, with the exception that the decolorized slide is not counterstained with methylene blue but immersed for 15 seconds in a half saturated aqueous solution of picric acid. On the uniformly yellow background the red serpentine cords of pathogenic Mycobacteria can be seen with the low power lens of a microscope.

The addition of Tween 80 to the medium is harmful for the purpose of testing cord formation. Tween 80 is an agent which opposes cord formation, producing dispersed growth of Mycobacteria.

**Material**

An agreement was reached between the laboratory of the Minnesota State Department of Health in Minneapolis and the Tuberculosis Research Laboratory of the Anoka State Hospital in Anoka (referred to as "Anoka laboratory"), that the Anoka laboratory was to receive cultures of Mycobacteria cultivated in the laboratory of the Minnesota State Department of Health from the pathologic material and tested for pathogenicity by
guinea pig inoculations for the purpose of testing for cord formation in liquid medium at the Anoka laboratory.

The Anoka laboratory received 249 strains of Mycobacteria from the laboratory of the Minnesota State Department of Health, four strains were in duplicate. The 245 remaining strains were tested for cord formation. To avoid the possibility of the results of guinea pig inoculation data influencing the results of the cord testing experiments at the Anoka laboratory, the results of the guinea pig inoculations at the laboratory of the Minnesota State Department of Health were given to the Anoka laboratory after the work of cord testing at the Anoka laboratory had been concluded. (Except 40 strains which were inoculated into guinea pigs at the Anoka laboratory. See later).

From the Minneapolis strains, 192 were marked with the number and the name of the patient, we designated these strains with an "M"; 40 strains besides the number and the patient's name bore the mark "C"; eight strains had the mark "N," and five strains the mark "R."

Results

The Minneapolis M strains were isolated from tuberculous patients; they all produced typical cords and were classified as lethal. These strains caused tuberculosis in guinea pigs. C strains produced yellow pigment and abundant early growth with formation of heavy pellicles on the surface of the medium. The growth was dispersed, no cord formation was detected. The C strains were classified as saprophytic. As it was discovered later, the C strains, on the ground of their obvious saprophytic cultural properties, had not been inoculated into guinea pigs. To verify the cultivation results, these 40 strains were inoculated into guinea pigs at the Anoka laboratory. These strains did not cause death or morbid lesions in guinea pigs.

In both the M and C strains, representing 94.3 per cent of the State
Board of Health material, full agreement between cord formation of the strains and the pathogenicity tests in guinea pigs was reached.

The N strains, as we were later informed, were isolated at the laboratory of the Mayo Clinic, Rochester, from the sputa, gastric washings and urine of patients. The growth of these strains was relatively slow, without pigment formation, and resembled the growth of lethal strains. These strains were not lethal to guinea pigs and accordingly have been classified as non-pathogenic. The N strains showed in liquid medium some rudimentary cord formation and one of them (N-5) on the ground of its cord formation, was classified as pathogenic. This N-5 strain in guinea pigs, inoculated subcutaneously with 0.1 ml. of the liquid culture, produced considerable caseation of the lymphatic nodes but the animals were alive after three months of observation. The N strains were recognized as non-lethal, although their origin from the lethal strains was presumed. Strains of this group were most difficult to classify. It was difficult to determine the stage at which the structure of the cords indicate the loss of lethality, when some pathogenicity of the strain still remains.

The R strains uniformly produced cords and were classified as lethal. These strains were isolated from the sputa and gastric washings of patients with tuberculosis, and all were resistant to 10 micrograms of isoniazid. Two of these strains were lethal when inoculated subcutaneously into guinea pigs; the three others were not lethal to guinea pigs. These strains, inoculated intravenously into mice, were lethal to those animals (Karlson).

The true, fully developed cord of lethal Mycobacterium is a tight formation of parallel bacilli, produced by sliding growth of them. Even the oil immersion objective of a microscope can scarcely reveal separate bacilli in the cord. With the decrease in pathogenicity the structure of the cord becomes more and more loose, and in saprophytic strains the formation

**FIGURE 3:** Dispersed growth of the saprophytic acid-fast bacilli (Magnification 480X).
disappears, the growth being dispersed or assuming the form of loose heaps. Generally, when the oil immersion objective recognizes in the axis of a grown-up cord well separated bacilli, the lethality of the strain is lost.

No one actually knows the role of the strains of reduced pathogenicity in human pathology, but the harmlessness of these strains must be doubted.

The results of pathogenicity and lethality tests of our strains are summarized in Table I.

**Discussion**

As our experiments revealed all strains of tubercle bacilli lethal to guinea pigs as well the strains of reduced pathogenicity, which do not kill guinea pigs following the usual route of inoculation, produce well-formed cords. Between the strains of full pathogenicity (lethal) and the complete absence of pathogenicity (saprophytic strains) is the spectrum of variable pathogenicity. Guinea pig inoculation can differentiate between lethal strains and saprophytic ones, although there is no need to inoculate animals with these strains. The liquid medium recognizes these strains with the same accuracy as the animal inoculation. The strains of reduced pathogenicity on the ground of guinea pig inoculation would be differentiated by a bacteriologist as "non-pathogenic"; the pathologist finds in the animals, inoculated with these strains, considerable morbid lesions. The variable pathogenicity of the intermediate strains is reflected in the structure of the cords. The strains derived from lethal strains, such as BCG, H37Ra, our R strains, developed in patients treated with antibiotics or chemotherapeutics, or sometimes of unknown origin, and producing true cords in liquid medium, must be recognized as pathogenic. The lethality of Mycobacterium to guinea pig is easily lost, the cord formation is conserved due to the rest-pathogenicity remaining in the bacilli.

**CONCLUSIONS**

The pathogenicity of 245 strains of Mycobacteria was tested by means of guinea pig inoculations; in another laboratory the same cultures were

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**TABLE I**

<table>
<thead>
<tr>
<th>Cord formation (Anoka Laboratory)</th>
<th>Guinea pig inoculation. (Laboratory of the Minnesota State Department of Health)</th>
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</thead>
<tbody>
<tr>
<td>Cultures</td>
<td>Cultures</td>
</tr>
<tr>
<td><strong>M</strong> strains, cords present</td>
<td>192 Tuberculosis in guinea pigs 192</td>
</tr>
<tr>
<td><strong>C</strong> strains, cords absent</td>
<td>40 No tuberculosis in guinea pigs 40*</td>
</tr>
<tr>
<td><strong>N</strong> strains, cords absent</td>
<td>7 No tuberculosis in guinea pigs 7</td>
</tr>
<tr>
<td><strong>N</strong> strains, cords present</td>
<td>1 No lethal tuberculosis 1</td>
</tr>
<tr>
<td><strong>R</strong> strains, cords present</td>
<td>5 Tuberculosis in guinea pigs 2 Tuberculosis in mice 3</td>
</tr>
</tbody>
</table>

*Inoculated into guinea pigs at the Anoka Laboratory.
tested for their cord-forming ability in the liquid medium.

Both methods, the guinea pig inoculation and the cord-forming ability, recognized 192 lethal and 40 saprophytic strains with equal efficiency.

The two above mentioned groups of Mycobacteria represented 94.3 per cent of the strains tested.

The remaining material consisted of eight N strains showing rudiment of cord formation. These strains were not lethal to guinea pigs but caused considerable morbid lesions in them. The origin of these strains from the lethal ones was presumed.

Five isoniazid-resistant R strains formed true cords; they were classified as lethal. Two of these strains were lethal to guinea pigs; when injected intravenously, the other three were lethal to mice.

Acknowledgement: We desire to give our heartfelt thanks to Dr. Henry Bauer, Minnesota State Department of Health, for furnishing us the unknown cultures, and to Dr. Alfred Karlson, Mayo Clinic, Rochester, Minnesota, for the non-lethal strains of Mycobacteria.

CONCLUSIONES

Por medio de las inoculaciones al cuy, se himieron pruebas de patogenici-
dad de 245 cepas de micobacterias; en otro laboratorio los mismos cultivos fueron ensayados respecto de su tendencia a hacer cordoness en el medio líquido.

Tanto la inoculación al cuy como la tendencia a formar cordoness, correspondieron a 192 cepas le tales y 40 saprófitas con igual aptitud.

Los dos grupos arriba mencionados de micobacterias representan el 94.3 por ciento de las cepas estudiadas.

El resto del material consistió en 8 cepas que mostraron rudimentaria formación de cordoness. Estas cepas no son letales a los cuyes pero causaron lesiones patológicas considerables en ellos. El origen de estas cepas a partir de las letales se supuso.

Cinco cepas resistentes a la isoniacida formaron verdaderos cordoness; éstas fueron clasificadas como letales. Dos de estas ce pas fueron letales para los cuyes; cuando se inyectaron intravenosamente las otras tres fueron letales para los ratones.

RESUME

La virulence de 245 souches de "mycobacterium" fut éprouvée par l'inoculation au cobaye. Dans un autre laboratoire, les mêmes cultures furent testées en fonction de leur tendance à se disposer sous forme de cordoness en milieu liquide.

Les deux méthodes, inoculation au cobaye, et possibilité de formation de cordoness, mirent en évidence avec la même efficacité 192 souches mortelles, et 40 souches saprophytes.

Les deux groupes de "mycobacterium" mentionnés représentèrent 94,3% des souches testées.

Le reste consistait en 8 souches N montrant une tendance à se disposer en cordoness. Les souches ne furent par mortelles pour le cobaye, mais pro-
voquèrent d'importantes lésions. On peut supposer que ces souches dérivaien des souches mortelles.

Cinq souches R résistantes à l’isoniazide formèrent de véritables cordes. Elles furent classées comme mortelles. Deux de ces souches furent mortelles pour le cobaye; lorsqu’elles furent inoculées par voie intraveineuse, les trois autres furent mortelles pour la souris.

SCHLUSSFOLGERUNGEN

Die pathogenen Eigenschaften von 245 Stämmen von Mycobacterien wurden mit Hilfe von Meerschweinchenimpfungen geprüft; in einem anderen Laboratorium wurden die gleichen Kulturen auf ihre strangbildende Fähigkeit im flüssigen Nährboden geprüft.

Beide Methoden, die Meerschweinchenimpfung und die Fähigkeit zur Strangbildung, liessen 192 letale und 40 saprophytische Stämme mit gleicher Wirksamkeit erkennen.

Die beiden eben erwähnten Gruppen von Mycobacterien stellten 94,3% der geprüften Stämme dar.

Das übrige Material bestand aus 8 N-Stämmen mit Anzeichen rudimentärer Strangbildung.

Diese Stämme wirkten auf Meerschweinchen nicht letal, verursachten wohl aber beträchtliche krankhafte Veränderungen bei ihnen.

Die Herkunft dieser Stämme von letalen Stämmen wird vermutet.

5 INH-resistente Stämme bildeten echte Stränge; sie wurden als letal eingeordnet. 2 dieser Stämme waren für Meerschweinchen letal; bei intravenöser Injektion waren die 3 anderen für Mäuse letal.

BIBLIOGRAPHY


