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Antigenic Properties of Malleomyces pseudomallei. I. Morphological, cultural, biochemical characteristics and variations of immunological type.

By L. Chambon and J. Fournier

Stanton and Fletcher (1), then Legroux and Genevray (2) have described two classical aspects of culture of the Whitmore Bacillus:

1. Mucoid or "grasses" colonies formed of entirely strained bacilli.

2. "Corrugated" or "folded" colonies formed of short germs, with bipolar staining.

This last form produces a film in liquid medium. It is biochemically more active and represents the most virulent phase, which one most often encounters on isolation.

Nicholls (3) has compared this dissociation to the variation S-R and has given a very precise description of numerous intermediary forms.

The "folded" colonies which one observes on isolation of the pathogenic products are now, however, "rough" colonies, for they are in general easily emulsified in saline and can give homogeneous suspensions with Millon reagent and trypaflavine. Furthermore, the N. O. P. strain (non-oxalate productive) of Nicholls (loc. cit.), obtains after aging in the presence of Cl_2Ca and glucose in gelatine broth may appear as form S and Form R: these two forms are not very virulent, which seems to indicate that virulence is not necessarily linked to the S or R aspect of the cultures.

The "grasses" colonies may be mucous and drawn out in fine filaments when sampled after inoculation; others, mor opaque, present a consistency like butter.

These facts are explainable if one takes into consideration the addition of somatic antigens O and R, two other antigenic factors whose role is preponderantly in the determination of characteristics linked to different phases of M. pseudo-mallei.

A. - Mucous antigen of Malleomyces pseudo-mallei.

On nutritive gelatin, the aging of colonies of the Whitmore bacillus is shown by opaqueness and by the appearance of a more or less mucous consistency. In peptone broth there is formation of a mucous deposit dissociated with difficulty.

Development of this mucous state is maximum on Koser medium with the addition of 1 p. 100 Bactopeptone, 10 p. 100 saccharine and 2½ p. 1000 gelatine; after three or four days of culture at 30°, one sees forming a large border of translucent mucous around the lysed and folded colonies which becomes yellowish and fluid.

Staining practised on smears of these colonies show lysed germs encapsulated in an amorphous substance. This aspect is particularly clear after Giemsa staining: the bacillary bodies are deformed, pale or reduced to the state of granules; the mucous membrane covering is slightly stained in violet.

Certain strains are spontaneously very mucous on nutritive gelatine and give birth, in peptone broth, to viscous filaments which are developed from the surface of the liquid.

The "suicidal cultures" of Nicholls (loc. cit.), which appear by disociation of a culture in phase S and which Le Gac (4) signals as frequent for a strain isolated from a patient treated with chloramphenicol, are characterized by translucid colony plates; the bacteria are equally lysed and reunited by a mucous substance.

The mucous substance which is formed under conditions just described and which correspond to lysis of the Whitmore bacillus is an antigen which

can be shown serologically, as we shall demonstrate later.

B. - Envelope Antigene of Malleomyces pseudo-mallei.

A culture on glucose broth, 1 p. 100, of a lysed and virulent strain of M. pseudo-mallei is inoculated on gelatine glucosed 0.1 p. 100. After incubation of 18 hours at 37°, the lysed colonies, examined by oblique transillumination shows two different aspects:

a) White opaque colonies

b) Clear bluish-gray colonies.

Both are lysed and regularly rounded. The former are more convex, brilliant, of fatty consistency and are not drawn out on inoculation. There is a scale of transition forms between typical a and b colonies. After 48 hours in the oven at 37°, all colonies become opaque and their center takes on a yellowish tint.

The particular aspects of bacterial morphology correspond to this dissociation. The technique of staining capsules with toluidine blue, indicated by Dumas (5) shows in colonies of type a a majority of the germs surrounced by colorless, well-defined envelope of variable thickness, sometimes a simple film difficult to distinguish, sometimes a mass in diameter appearing eight times larger than that of the stained stroma. (fig. 1)

Colonies of type b contain only a weak proportion of bacteria provided with an envelope: their secondary opaqueness by aging does not increase the number of such individuals.

In colonies of an intermediary type between a and b, one observes variable proportions of germs surrounded by an envelope.

This dissociation is independent of the dissociation "grasse" → "folded". The folded colonies may be clear or opaque: the latter are formed of short bacteria with bipolar staining and surrounded by an envelope.

On gelatine Koser medium, the appearance of opaque colonies is favored by certain glucides, in particular by glucose (lysed colonies), glycerine and sucrose (folded colonies).

This envelope is not a true capsule. Examination in the fresh stage in phase contrast shows, in effect, that the bacteria which are provided with one may be perfectly mobile and we have not been able to provoke the phenomenon of swelling of the capsule with experimental sera.

This appearance of the capsule has not escaped observation of numerous authors, in particular Pons (6), Nicholls (loc. cit.) and Miller (7), without, however, having shown the difference in nature which exists between the envelope antigen of opaque colonies and the mucous antigen of bacterial colonies being lysed.

To what does this appearance of the capsule respond?

Examination of the Whitmore bacillus by electronic microscope has enabled Miller (loc. cit.) to describe, alongside opaque corpuscules resembling dense protoplasmic accumulations, clear, dispersed globules of lipoid aspect, which are detached from the bacillary bodies under the effect of sonic vibrations.

According to Finlayson (8) these globules, stained with phenicated fuchsian are decolored by alcohol but resist decoloration by acids.

We think that, under favorable conditions and in particular with certain strains recently isolated from pathological products, that it may concern forms S. or R, these globules may take such development that they completely surround the bacteria, giving thus an appearance of capsule. We shall show later that this substance of the envelope can be demonstrated serologically.

C. - Characteristics of difference phases of Malleomyces pseudo-mallei.

If one indicates by M the mucous antigen, by K the envelope antigen, by O and R the somatic antigens, one can theoretically imagine a great number of combinations of these four factors. We will limit ourselves to the description of forms currently observed.

1. Types habitual to isolation . -- On isolation, hemocultures and pyocultures generally give folded colonies, opaque, and of a metallic luster, more rarely smooth and opaque colonies. The former correspond to the form R K with short bacteria and bipolar staining; the latter to form O (R) K with short germs, entirely stained. In both cases there is an envelope clearly visible.

Form R K is stable. It has glucidolytic activity, and high proteolytic and hemolytic activity; it reduces neuter red and methylene blue and gives a weak quantity of H_2S in gelose to lead subacetate; it utilizes the C of citrate of Na on Simmons medium, hydrolyzes urea in the presence of a glucide and reduces nitrates in nitrites.

Form O (R) K is on the contrary unstable. From the first transplant, it gives on nutritive gelose a large number of clear, gray-blue colonies, where the germs are rarely provided with an envelope plainly seen and which represents the form O (R) K minus. There exists also form R K minus. These two forms are characterized by a decline in their glucidolytic, proteolytic and hemolytic activity and reduction much larger than dissociations O (R) K $\rightarrow$ O(R)Km and RK $\rightarrow$ RKm are more important. Fermentation of glucides in the course of these dissociations is more a question of degree than of nature; however, sugars fermented by the most dissociated strains are, in the order of frequence, glucose, galactose, maltose and levulose.

2. Habitual type of old strains of collection. -- This is the form O(R) K minus characterized by lysed colonies, gray-blue, clear, regularly rounded, where bacteria are of a median length, the majority entirely stained and rarely provided with a clearly visible envelope.

3. Strain obtained after passage in bile broth. -- After several bi-weekly transplants in bile broth, 50 p. 100, the C 141 strain of Whitmore bacillus presents the following characteristics:

Cultural characteristics. — In nutritive broth the culture is slow and

not abundant; the broth is lightly marked with formation of without appearance of scum.

On nutritive gelatin, growth is equally slow. After 18 hours in the oven at 37P, the colonies are very fine and transparent; they attain their full development after 30 hours of culture and appear then in the form of colonies of 0.5 with 1mm diameter, clear, gray-blue, easily emulsified in saline. Following, they become opaque and of a dirty yellow color.

These culture in broth and on gelatine are devoid of the characteristic aromatic odor of M. pseudo-mallei.

Morphological characteristics. — The bacteria are polymorphic, their cytoplasm is oven irregularly stained; they are devoid of envelope.

Biochemical characteristics. — This strain ferments glucose galactose with secondary alkalization, maltose slightly, it is neither proteolytic nor hemolytic; it does not use the C of citrate of Na and does not reduce neuter red; it hydrolyses with urea in presence of a glucide and reduces nitrates into nitrites.

Pathogenic Potentiality. — It is not pathogenic for the guinea pig by intraperitoneal route in a dose of 5.10^5 germs, whereas under the same conditions, the DL_{50}(1) of a culture of an opaque colony of strain C 141 is 158 germs.

While proceeding to the study of its antigenic constitution, we class this strain avirulent, smooth and devoid of envelope to the extreme limit of variation OK → OKm and we indicate by O (k) on fig. 1.

4. Strain R. — This strain has been obtained by culture of the strain O (k) in presence of serum anti-O.

On nutritive gelatine it gives parchmented colonies, flat round, with regular or irregular borders, flat or slightly raised, folded, surface or slightly ridged, translucent and yellowish. These colonies are adherent to gelatin, then give the phenomenon of moving off in bulk: they are not emulsified

in physiological saline.

The culture on nutritive broth is characterized by the formation of a microbial accumulation which appears after 8 hours of culture at 37°, then is reunited in a thin veil, translucid, folded, and indissociable, on clear liquid.

The bacteria are seen in the form of bacilli and diplobacilli, long, entirely stained by analine strains, devoid of envelope.

This strain presents no glucidolytic, proteolytic and hemolytic activity; it reduces neuter red but not methylene blue; it utilizes C of citrate of Na and dydrolyses urea in presence of glucide; it reduces nitrat s in nitrites.

It is not pathogenic for the guinea pig by intraperitoneal route.

CONCLUSIONS

The different forms under which cultures of M. pseudo-mallei occur do not enter into the limits of the variations S → R

(1) This DL_{50} has been determined by the method of total cumulation, after four days' observation of the animals.

Colonies smooth and plissees may be opaque or clear, depending upon whether the bacteria of which they are composed are provided with an antigen envelope K. Furthermore a mucous antigen M may appear in the course of bacterial lysis.

We shall eventually bring serological proof of the existance of antigens K and M. But we can envisage variations of the immunological type pf M pseudo-mallei in the following manner:

$$RK \rightarrow RKm \leftarrow O(R)Km \rightarrow O(R) \rightarrow R$$

$$\text{et } O(R)K \rightarrow O(R)Km \rightarrow O(R) \rightarrow R,$$

the forms R K and O (R) K being seen on isolation from pathological products and the others in the course of subcultures.

We have never had occasion to observe form O (R) pure, of which a strain obtained after culture in bile broth seems to most similar: it is in cultivating this strain in presence of serum anti-O that we obtained a strain R.

The appearance of forms O in the course of the variation R K —— R does not conform to the classically admitted facts. The appearance of smooth forms from folded forms is, however, currently observed in the course of transplants or after conservation of strains in maceration of jellied meat. Forms R K and R K M are not perhaps entirely deprived of antigen O. This fact remains to be verified by serologic proof.

Antigen M can be present in all forms.

We have summarised in table I the modifications of characteristics of B. pseudo-mallei as a function of the variations of immunological type. One cannot describe fixed characteristics of the form O (R) K which is immediately dissociated. On the whole, biochemical activity would appear in direct relation to the presence of antigen K; its progressive disappearance in the course of transplants and aging of strains takes account of the classic diminution described of this activity. It is the same for virulence.