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Antigenic Constitution of Malleomyces Pseudo-mallei.

II. Demonstration of existence of antigens M, K and O and Study of their Immunologic Properties.

by L. Chambon and J. Fournier

We have previously seen that the characteristics of different phases of Malleomyces pseudo-mallei would appear to be determined by differing associations of four antigenic factors: the mucous antigen M, envelope antigen K, somatic antigen O and antigen R.

In the absence of forms S or R entirely deprived of antigen M or K, we have prepared specific agglutinating serum by using the differing action of certain physico-chemical agents on each one of these antigens.

A- Techniques.

a. Preparation of microbial suspensions. — Tubes of nutritive gelose slants containing glucose of 0.1 p. 100 are seeded with a culture in glucose broth, 1 p. 100, of the strain of Whitmore bacillus C 141, smooth and virulent. After 18 hours in the oven at 37°, the culture, characterized by numerous opaque colonies, is harvested on nutritive broth. Roux boxes, containing 250 ml each of nutritive gelatin, are seeded with this suspension, 2 ml per box. The harvest is effected in saline after 18 hours of incubation at 37°.

Formol suspension. — A suspension in saline containing 2.10^9 germs, (washed three times), per millilitre, is submitted to the action of formol in a concentration of 0,5 p. 100 for 20 hours at 37°.

Suspensions heated at 60°, 100°, 120°. — Three suspensions in saline containing 20.10^9 germs, washed three times, per millilitre are respectively submitted to a heating at 60° for one hour, to 100° for two hours and a half, and to 120° for two hours. They are then washed two times and formolated at 0,5 p. 100.

Suspensions treated with ethyl alcohol at 50 and 96 p. 100. — Two suspensions, one in 50 p. 100 of ethyl alcohol, the other in 96 p. 100 ethyl alcohol, each containing 20.10^9 germs per millilitre, washed once, are incubated for 20 hours at 37°. They are then washed two times and formolated at 0,5 p. 100.

Suspension treated with normal chlorhydric acid. — Germs washed once are put in suspension in distilled water so as to have 40.10^9 g/ml. A quantity of chlorhydric acid 2N is added equal to that of distilled water. Thus is obtained a suspension of 20.10^9 germs p r millilitre of chlorhydric acid N. This suspension is put in a balloon flask containing glass bits and left in the oven at 37° for twenty hours, being agitated from time to time. The germs are then submitted to two careful washings with complete dissociation of the agglomerates, then formolated at 0,5. 100.

For the preparation of all these antigens, the washings are effected in saline. The final suspensions in saline, containing 20.10^9 g/ml and formolated at 0,5 p. 100 are decanted in 24 hours to 4° which permits elimination of a residue formed of inassociable agglomerates. They are then kept at 4°. The suspension treated by N-HCl is unstable at 37°.

b. Chemical extraction of antigen O. — This concerns a trichloracetic extract prepared following the technique of Boivin and Mesrobeanu (1).

(c) Preparation of agglutinating serum.—1st. The preparation of microbial suspensions.—The suspensions for which we have just indicated the preparation, are diluted in saline so as to obtain a density equal to 500.10^6 germs per millilitre.

Each of them is injected intravenously into a rabbit in doses of 0,5, 1, 1,5, 2 and 2,5 ml, all the five days.

2nd. For trichloracetic extract.—Inject intravenously into the rabbit 10 ml of a solution of undetermined concentration of trichloracetic extract in five injections made at four day intervals.

The rabbits are bled a week after the last injection.

Serums thus prepared are increased by 0,01 p. 100 of merthiolate and kept at 4^o.

(d) Reactions of agglutination and absorption of agglutinins.—The reactions of agglutination are made by centrifugation and on slides. In the first case, X drops of microbial suspension are mixed with X drops of dilution of serum and the reading is made after five minutes of centrifugation at 2000 t/m. In the second, mixtures containing I drops of microbial suspension and I drops of dilution of serums and the reading is made after an agitation of 4 to 20 minutes on a Kline agitator.

Results are noted in the following manner:

Centrifugation. ++++ : agglutinants in a clear liquid

+++ : agglutinants in a troubled liquid.

++ : Agglutinants visible to the naked eye.

+ : agglutinants visible with magnifying glass

On Plate. The number of signs + (of ++++ to +) is a function of the intensity of the reaction.

Absorption of agglutinins is made following two techniques:

saturation of serums by very dense microbial suspensions in the course of two hours at 37° and eighteen hours at 4°; the quantitative technique of Felix and Pitt (2).

In the course of our exposition, we will designate microbial suspensions treated with formol at 0,5 p. 100, alcohol 50 p. 100, alcohol 96 p. 100, heat at 60°, 100°, 120°, and N-HCl, by G F, G Al_{50}, G Al_{96}, G 100°, G 120°, G N-HCl and the corresponding agglutinating serums as S. G F, S. G Al_{50}, S. G Al_{96}, S. $G_{60°}$ S. $G_{100°}$, S. $G_{120°}$ and S. G N-HCl.

B. — Demonstration of the existence of antigens M, K and O.

Some reactions of agglutination are practical on slide and by centrifugation, in presence of G F, G Al_{96} and G N-HCl, with the following serums:

S. G F
S. G F saturated with G N-HCl : S. M K
S. M K saturated with G Al_{96} : S. M
S. G Al_{96}
S. G Al_{96} saturated with G N-HCl : S. K.
S. G N-HCl : S. O
Serum prepared with the extract T.C.A. : $S.O_2$

The results obtained are indicated in Table I.

Serum M agglutinates only the G F, the serum K agglutinates only G Al_{96}, and serum O_1 agglutinates G Al_{96} and G N-HCl into two nearly equal parts.

We propose to show that:

1. Agglutination of G F by S.M is an agglutination due to antigen M.
2. Agglutination of G Al_{96} by S.K is an agglutination due to antigen K.
3. Agglutination of G N-HCl by $S.O_1$ is an agglutination due to antigen O.

1. Antigen O. — The trichloracetic extract of a ciliated, gram-negative bacillus, such as Whitmore's bacillus, corresponds, according to Boivin and Mesrobeanu, to the somatic antigen O. We will consider then, that the serum O_2 is an anti-O serum.

To show the identity of agglutinins of serums O_1 and O_2, we have saturated dilutions in geometric progression 2 of these two serums by determined quantities of G N-HCl and extract T. C. A. until the disappearance of the agglutinins anti-G N-Hcl.

The technique is indicated in Table II.

Results obtained show that the agglutinins anti-G N-HCl of serums O_1 and O_2 are, at equal titer, saturated with comparable quantities of G N-HCl on one part, and extract T.C.A. on the other part. These agglutinins are, then, identical and correspond to antigen O of Whitmore's bacillus.

2. Envelope Antigen K. — After the preceding results, agglutination of G N-HCl by S. G Al_{96} is an O agglutination; it disappears after saturation of this serum by G N-HCl, whereas there persists an agglutination a 1/640 by centrifugation and at 1/320 on slide for G Al_{96}.

Thus is established the existence of an antigen destroyed by N-HCl and different from antigen O. One can suppose that it concerns envelope antigen K; it determines, actually, as we shall see elsewhere, an O-inagglutinability which is a function of dissociation O (H) K $\longrightarrow$ O (R) K m.

3. Mucous Antigen M. — It appears on Table I that the G F are not agglutinated with by S, K or by S. O_1. This K- and O-inagglutination is determined by a third antigen which is mucous antigen M.

After saturation of 3. CF by G N-HCl to eliminate the agglutinins O (S.MK), then by G Al_{96} to eliminate agglutinins K, S.M is obtained, which agglutinates G F at 1/512 by centrifugation and at 1/256 on slides.

4. Conclusion.— Obtaining extracts M and K by chemical means would permit establishing their relation to serums M and K with the aide of reactions of inhibition of agglutination comparable to those used for antigen O. Attempts at extraction are in progress.

We can, however, taking as a basis the inagglutination which results from the position of antigens M, K and O in the bacterial cell, conclude that three antigens enter into the constitution of Malleomyces pseudo-mallei. These are, moving from the periphery toward the center:

a) Mucous antigen M, unaltered by formol at 0.5 p. 100, destroyed by ethyl alcohol at 96 p. 100 and by normal chlorhydric acid;

b) Envelope antigen K, not altered by formol at 0.5 p. 100 nor by ethyl alcohol at 96 p. 100 but destroyed by normal chlorhydric acid;

c) c) Somatic antigen O, resistant to normal chlorhydric acid and similar to that of Gram O negative bacteria.

We can thus, up to the present, interpret the results of Table I in the manner shown by Table III.

The existance of the three antigens M, K and O being established, it remains for us to characterize them more precisely while studying the behavior of their antigenic capacity, their fixative power and their agglutinability after treatment with ethyl alcohol at 50 p. 100 and 96 p. 100, with N-HCl and after heating at 60°, 100°, and 120°.

C. — Properties of Antigen K.

1. Agglutinability. — Agglutination by centrifugation and on plate of suspensions of Whitmore's bacillus:

Treated with alcohol 50%	G Al_{50}
Treated with alcohol 96%	A Al_{96}
Heated 2½ hrs. at 100°	G 100°
Heated 2 hrs. at 120°	G 120°
Treated with N-HCl	G N-HCl

serum G Al_{96} saturated with G N-HCl (S.K (Al_{96}), gives the results indicated in table IV.

By centrifugation, one obtains with suspensions of G Al_{50}, of G Al_{96}, and especially those of G 100° and G 120°, a "crepe" agglutination, comparable to that of the surface antigen of E. coli. These four suspensions are equally agglutinated (1/640).

The technique of agglutination on slides is less sensitive, in particular for G 100° and 120°; it is characterized by agglutinates which should appear after eight minutes of agitation.

2. Fixative power of agglutinins. The fixative power of agglutinins of G Al^{50}, G 100° and G 120° is evaluated by reference to that of G Al_{96} by quantitative saturation of serum K (Al_{96}) with a titer equal to 1/320 (on slides)

Results of tables V and VI show that the minimum quantity of G Al_{96}, G Al_{50}, G 100° and G 120° necessary to saturate the agglutinins of this serum is equal to 4.10^9 germs.

The fixative power of agglutinins K of G Al_{96}, G Al_{50}, G 100° and G 120° is then identical. (Tables V and VI)

3. Antigenic Capacity. — Serums K are prepared by saturation of S.GF, of S. G Al_{50}, of S. G 100° and G 120° by G N-HCl. We will designate them thus: S. K (F), S. K (Al_{50}), S. K(100°) and S. K(120°).

These serums, the same as S. K(Al_{96}), agglutinate with G Al_{96} at the rate of 1/640 by centrifugation and 1/320 on plates (table VII).

The five suspensions possess an antigenic capacity nearly equal.

D. — Properties of Antigen M.

1. Agglutinability. — The fixative power of agglutinins of antigen K not being destroyed by heat of 100°, one can obtain a serum M devoid of agglutinins K while saturating S. G F with G 100°.

This serum does not agglutinate, by centrifugation or on slides, except with G F and G 60°. (Table VIII)

The best results are obtained using suspensions of G F, washed once, regulated at 1.10^9 g/ml to agglutinate by centrifugation and 2.10^9 g/ml to agglutinate on slide.

In a tube, one obtains agglutinates partially dissociable in a troubled liquid; on slide, they are large and floculent for even the weakest dilutions.

2. Fixative power of agglutinins. — The fixative power for agglutinins M of G Al_{50}, G Al_{96}, and G 60° is determined by reference to that of G F for an S. G F saturated with G 100°.

The technique used is the same as that used for antigen K.

The fixative power of agglutinins M is zero for G Al_{96}, strongly diminished for G Al_{50}, with reference to that of G F and G 60° (table IX).

3. Antigenic capacity. — A formolated suspension is agglutinated by centrifugation and on slides by S. GF, S.G Al_{50}, S.G Al_{96} and S.G 60° saturated by G 100°.

Results given for agglutinations show that G F and G 60° possess an equal antigenic capacity, since that of G Al_{50} is diminished and that of G Al_{96} is zero (table X).

E. — Inagglutinability of antigens K and O.

A reading of table I shows that serum K does not agglutinate a suspension of G F and that serum O_1 does not agglutinate this suspension, although it agglutinates suspensions of G Al_{96} and G N-HCl.

These results lead us to study the conditions under which antigen M determines a K and an O-inagglutinability, and antigen K an O-inagglutinability.

1. <u>K- and O-inagglutinability due to antigen M.</u> — For this study we use a serum K deprived of agglutinins M, such as K(Al_{95}) and a serum O prepared with G N-HCl.

Suspensions of GF, G Al_{50}, G N-HCl are agglutinated comparatively by these serums K and O, and by a serum M.

The results of table XI show that antigen M determines a K- and an O-inagglutinability.

2. <u>O-inagglutinability, due to antigen K.</u> — On the other hand, it seems that antigen K does not determine O-inagglutinability; suspensions of G Al_{96} and of G N-HCl are in effect agglutinated at an equal titer by serum O.

The influence of the presence of antigen K on the inagglutinability of antigen O depends, really, on the importance of the dissociation O K $\longrightarrow$ O K m $\longrightarrow$ O.

We give as example the results we have obtained in agglutinating by an S. G N-HCl microbial suspensions prepared from a culture very dissociated (O K m) and one less dissociated (O K) (Table XII).

A culture slightly dissociated my be O-inagglutinable under the form of a suspension of G Al_{50} and hypoagglutinable under the form of a

suspension of G Alg$_E$ or of G heated. One observes as well, the existence of a phenomenon of sons with the germs O K m treated with alcohol of 50 p. 100.

Agglutination O is granular; it is slow on slides where it is maximum after 20 minutes of agitation.

The fixative power of agglutinins O of these suspensions is inversely proportional to their O-inagglutinability.

Their antigenic capacity is about the same, as indicated in table XIII.

Conclusions

The study of the different culture types of Malleomyces pseudomallei suggests the existence of three antigens: a mucoid antigen M, a capsular antigen K and a somatic antigen O.

The authors have demonstrated their existence by serological tests based upon their difference in sensitivity tothe action of formol, ethyl alcohol, heat and normal hydrochloric acid and upon inagglutinabilities caused by these antigens.

The properties of the M antigen of Whitmore's bacill s are similar to those of the M antigen of the Enterobacteriaceae. This antigen produces an agglutination which may be partially dissociated in tubes and appears rapidly on slides. It causes K and O-inagglutinability.

Antigen K behaves like a capsular antigen. It gives a "crape type" agglutination on centrifugation, total and immediate on slides. It determines an O-inagglutinability or hypo-agglutinability variable according to the extent of the dissociation OK→OKm→O, which can be total with a bacterial suspension treated with 50% alcohol.

The somatic antigen O is comparable to that of the Gramnegatives. It gives granular agglutination by centrifugation, tardy on slides.

TABLES

Table II	Inhibition of agglutination of G N-HCl by serums O_1 and O_2, after saturation of agglutins by G N-HCl (R_1) and by an extract T.C.A. (R_2)/
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Table XIII	Antigenic capacity of antigen O.
Table XIV	Sensibility of antigens M, K and O to physico-chemical agents.

Note... Other tables ... no translation.

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