STUDIES ON THE RELATIONSHIP OF CERTAIN VARIANTS OF B. TYPHOSUS

I. Agglutination and Agglutinin Absorption

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Serological analyses of the constitution of the motile bacilli have established the existence of a heat-labile agglutinogen associated with the flagellae and of a heat-stable agglutinogen contained in the bacillary bodies. Whereas there appears to be no antigenic difference in the flagellar substance of the smooth and the rough forms, the studies of the somatic antigens of these variants have not led to clean-cut results, although the tendency has been to consider them entirely dissimilar.

The purpose of the present paper is to present the results of cross-agglutination and agglutinin absorption tests, using the four known variants of *B. typhosus*, namely the motile smooth, non-motile smooth, motile rough, and non-motile rough forms, and to suggest a formula for the antigenic composition of these strains.

As a result of the work on the dissociation forms of various motile bacilli by Malvoz (1), Smith and Reagh (2), Orcutt (3), and Walker (4) dealing with the flagellar and somatic antigens and by Weil and Felix (5), Arkwright (6), Gardner (7), White (8), Li (9), and others working with the rough and smooth, as well as with the motile and non-motile forms, the following antigenic fractions have been assumed to be present in these organisms: (1) the flagellar antigen H; (2) the smooth body antigen O; (3) the rough body antigen Ø or R. Thus, the smooth motile form (MS) would have the antigenic composition HO; the smooth non-motile form (NS) the composition O; the rough motile form (MR) the composition HØ; and the rough non-motile form (NR) the composition Ø. There would, therefore, be no somatic antigen common to the smooth and the rough variants.

The theoretical results of agglutination tests based on this assumption would be that the anti-MS serum would agglutinate all but the NR antigen and the anti-MR serum would agglutinate all but the NS antigen, while the anti-NS and anti-NR sera would agglutinate only the smooth and the rough strains respectively.

Li, working with these four variants of the hog cholera bacillus, has reported results in close agreement with this theoretical scheme of agglutination, except that his anti-NS (O) serum agglutinated the MR (HØ) antigen, although they presumably contain no common antigenic factor.

Gardner's actual experiments with *B. typhosus* do not correspond quite so closely with the theoretical results since his anti-MR serum agglutinated the NS culture and his anti-NR serum agglutinated both the MS and the NS cultures. These differences, however, he believes to be due to the fact that he used for the NS and NR antigens boiled suspensions of the corresponding motile forms and that the boiling not only destroyed the flagellar antigen but tended to change the smooth somatic (O) antigen to the rough (R) form.

In the course of a test to determine the immunological response to the usual typhoid vaccination with smooth and with rough vaccines, the writer (10) found that the sera of three individuals receiving the rough vaccine, containing both MR (HØ) and NR (Ø) forms, agglutinated an NS (O) culture, and that the sera of three individuals receiving the smooth vaccine, containing both MS (HO) and NS (O) forms, agglutinated an NR (Ø) culture.

Since these tests indicated that the smooth and rough variants of *B. typhosus* possess a common somatic agglutinogen, a fact not in accord with the conception of antigenic composition outlined above, it was clearly necessary to confirm the results by agglutination and agglutinin absorption tests, using each of the four variants (MS, NS, MR, and NR) in pure culture.

EXPERIMENTAL

The variant strains used in the following experiment were derived from two cultures. The motile smooth strain (MS) and the non-motile smooth strain (NS) were obtained from motile and non-motile colonies of a culture freshly isolated from a case of typhoid fever. The motile rough strain (MR) and the non-motile rough strain (NR) were from a stock strain of *B. typhosus* (Rawlins). The following characteristics distinguished the four variants:

MS: Surface colonies on agar plate—smooth. Deep colonies in semisolid agar—large and irregular. Motile. Virulence for mice—0.01 cc. to 0.001 cc. of 18 hour broth culture. Number of organisms killed by 0.5 cc. of a 1/12 dilution of human blood—12 to 1,200.

NS: Surface colonies on agar plate—smooth. Deep colonies in semisolid agar—small and compact. Non-motile. Virulence for mice—0.01 cc. to 0.001 cc. of 18 hour broth culture. Number of organisms killed by 0.5 cc. of a 1/12 dilution of human blood—12 to 120.

MR: Surface colonies on agar plate—rough. Deep colonies in semisolid agar—large and irregular. Motile. Virulence for mice—1.0 cc. of an 18 hour broth culture. Number of organisms killed by 0.5 cc. of a 1/12 dilution of human blood—1,200,000.

NR: Surface colonies on agar plate—rough. Deep colonies in semisolid agar—small and compact. Non-motile. Virulence of mice—1.0 cc. + of an 18 hour broth culture. Number of organisms killed by 0.5 cc. of a 1/12 dilution of human blood—1,200,000.

The virulence of the NS culture for mice was usually somewhat greater, and never less than that of the MS culture. The NR culture was slightly less virulent than the MR culture.

In preparing the sera for the animal experiments, the rabbits were given seven intravenous injections of 0.5 cc. of the vaccines MS, NS, MR, and NR respectively at 7 day intervals, and were bled 9 days after the last injection. The antigens for the agglutination tests were the same suspensions that were used for producing the respective sera, but were diluted with three parts of broth for the agglutination tests.

The agglutinations were carried out by adding 0.5 cc. of a diluted formalinized broth culture to 0.5 cc. of the serum dilutions. The reactions were read after 2 hours in the water bath at 53°C. and again after 18 hours in the refrigerator. The suspensions used for absorbing the sera were 24 hour cultures on hormone agar in Kolle flasks washed off with normal saline, heated to 56°C. for 1 hour, divided into the requisite number of tubes, and centrifuged.

For absorption 2 cc. of a 1/10 dilution of the serum were added to the growth from one Kolle flask, incubated for 1 hour in the water bath at 53°C., centrifuged and the serum added to the growth from a second Kolle flask, returned to the water bath for an hour, and allowed to stand overnight in the refrigerator before centrifuging. Further absorption failed to reduce the titre of the sera.

The four antigens were controlled with normal rabbit serum, and showed no agglutination in dilutions of 1/100 and over.

From Table I, Column 2, it is seen that not only does the anti-MS serum agglutinate the NR culture and the anti-MR serum agglutinate the NS culture, but the anti-NS serum agglutinates the NR culture and the anti-NR serum agglutinates both the MS and NS cultures.

That the weak agglutination of the motile rough culture by the sera prepared with the non-motile antigens is not due to failure of antigen-antibody union is shown by the fact that the motile rough

TABLE I

Cross-Agglutination and Agglutinin Absorption Tests of Cultures MS, NS, MR, and NR with Anti-MS, Anti-NS, Anti-MR, and Anti-NR Sera

1	2	3	4	5	6
Antigen	Serum unabsorbed	Serum absorbed with culture			
		MS	NS	MR	NR
		Serum MS			
MS	12,800 F	6,400 G	12,800 F	6,400 G	6,400 F
NS	6,400 G	0	0	0	0
MR	1,600 F	0	1,600 F	0	1,600 F
NR	3,200 G	0	0	0	0
		Serum NS			
MS	1,600 G	0	0	0	0
NS	3,200 G	0	0	0	0
MR	200 G	0	0	0	0
NR	3,200 G	0	0	0	0
		Serum MR			
MS	12,800 F	6,400 G	12,800 F	6,400 G	6,400 F
NS	3,200 G	0	0	0	0
MR	6,400 F	0	6,400 F	0	6,400 F
NR	3,200 G	0	0	0	0
		Serum NR			
MS	1,600 G	0	0	0	0
NS	1,600 G	0	0	0	0
MR	200 G	0	0	0	0
NR	3,200 G	0	0	0	0

F = flocculent agglutination.
G = granular agglutination.
O = no agglutination in a dilution of 1/100.

culture absorbs from these sera all the agglutinins for the three other cultures. 24 hour motile rough cultures agglutinate only slightly with the anti-NS and anti-NR sera, whether living, formalinized, or

killed at 56°C.; but 3 hour cultures agglutinate to titre whether living, formalinized, or heat-killed, as do the 24 hour cultures after heating to 75°C. for 30 minutes.

The cross-agglutination experiments here recorded were repeated on a number of occasions. 24 hour living broth cultures were substituted for the formalinized suspensions and sera prepared with boiled cultures of the motile strains were used in place of the anti-NS and anti-NR sera. The results, while varying slightly in agglutination titre, were always confirmatory of those given in Table I.

The absorption tests recorded in Table I show that whereas the non-motile cultures naturally fail to absorb the flagellar agglutinin from the anti-MS and anti-MR sera, each of the cultures completely removes the granular, somatic agglutinin from each of the sera, except that the MS culture still gives granular agglutination with the anti-MS and anti-MR sera after absorption with these cultures.

DISCUSSION

The cross-agglutination tests with the four variants of *B. typhosus* here reported can only be explained by assuming the presence of a common antigenic factor in the smooth and the rough forms, and the agglutinin absorption experiments confirm this assumption. The results reported by Li working with the same four variants of the hog cholera bacillus can readily be explained on the basis of the presence of three antigenic constituents, H, O, and Ø in different combinations in the four strains. Since the results here reported with *B. typhosus* cannot be explained on the basis of the presence of these three antigenic constituents, it would appear that one cannot generalize from the reactions of one member of the enteric group of organisms.

The possible objection that the cultures used in these experiments were not pure type variants was guarded against by numerous examinations of the cultures used in preparing the antigens. Both smooth and rough strains were examined on a number of agar plates, and the rough strains were further tested for the possibility of reversion to the smooth form by passage through mice and guinea pigs. Further, the rough strains were passed through twenty subcultures in 20 per cent homologous rough antiserum broth without increase in virulence or the appearance of smooth colonies. The non-motile strains were

examined microscopically and frequently inoculated into semisolid agar without showing any evidence of motility.

Although the results of the experiments here reported clearly point to the existence of a somatic agglutinogen common to the four variants, there are two phases of the tests which require further discussion; first, the weak agglutination of the motile rough culture by the sera prepared with the non-motile antigens, and second, the persistence of the granular agglutination of the motile smooth culture by the anti-MS and anti-MR sera after absorption by the MS and MR antigens.

That the failure of the formalinized MR culture to be agglutinated by the anti-NS and anti-NR sera is due to a physical interference with the granular agglutination by the flagellae and not to a failure of antigen-antibody union is shown by the fact that the MR culture absorbs from these sera the agglutinins for the other three variants, and by the fact that agglutination takes place if the flagellar agglutinogen of the MR culture is destroyed by heating the antigen to 75°C. for $\frac{1}{2}$ hour. Furthermore 3 hour motile rough cultures, whether living, formalinized, or killed by heat, agglutinate to titre with the anti-NS and anti-NR sera. Although it is not clear why the young cultures agglutinate while the older cultures do not, it might be assumed that this condition is due to a relatively slow development of the flagellar antigen in the young cultures.

The persistence of the granular agglutination of the MS antigen by the anti-MS and anti-MR sera after absorption with the MS and MR cultures cannot well be explained as a non-specific effect since not only do these absorbed sera not agglutinate the MR antigen; but the MS antigen is not agglutinated by normal rabbit serum nor by anti-NS nor anti-NR serum when absorbed by any of the four variants. The possiblity that the granular agglutination was due to insufficient absorption was guarded against by absorbing four times with a heavy suspension of MS followed by two absorptions with the dilute suspension used for antigen in the agglutination tests without reduction of the titre of the serum. Absorption with heavy suspensions of living MS culture likewise failed to remove the granular agglutinin, as did absorption with MS followed by absorption with NS. Therefore no explanation for the persistance of the granular agglutination in these cases has as yet been found.

To explain the results one must assume the presence of a common somatic antigen in the four variants of *B. typhosus* and of a flagellar antigen common to the two motile forms. Analyses, as yet incomplete, would indicate that the common somatic antigen is connected with the body protein of the bacilli, and that the factor which differentiates the smooth from the rough forms is the carbohydrate fraction which does not act as an agglutinogen. Whether the difference between the smooth and the rough variants is due to the presence of two different carbohydrates, S and R, or to a difference in the amount of S in the two forms is as yet undetermined.

Since the initials H (*Hauch*) and O (*ohne Hauch*) first used by Weil and Felix to describe the appearance on agar of the motile and non-motile forms of *B. proteus*, have no significance when applied to *B. typhosus*, and since the designations O and Ø (or R) applied by other writers to the smooth and rough antigens do not appear, in the case of *B. typhosus*, to designate different substances but merely the same constituent combined in one case with the soluble specific substance (S) and in the other either with a small amount of S or with the carbohydrate of a rough form, the following fractions are suggested as composing at least the more important antigenic constituents of the four variants of *B. typhosus:* P, the somatic antigen common to all four forms; F, the flagellar antigen of the motile forms; S, the soluble specific substance contained in the smooth forms; and R, the rough carbohydrate. The antigenic composition of the variants would then be as follows:

Motile smooth (MS)	= P + S + F
Non-motile smooth (NS)	= P + S
Motile rough (MR)	= P + F (+R)
Non-motile rough (NR)	= P (+R)

In this theoretical composition, the experiments here described establish the presence of the same somatic agglutinogen (P) in all four variants and give no experimental support to the previous theory that the smooth virulent organisms had an antigen O distinct from the substance Ø of the rough non-virulent organisms. The existence in the motile forms of a heat-labile flagellar agglutinogen F is confirmed; but the nature of the substance S to which the virulence of smooth

forms is due is merely suggested as analogous to the soluble specific substance of the pneumococcus. Preliminary tests have shown the presence of one or more carbohydrates in approximately equal concentration in the two forms; but whether there is a distinct carbohydrate R in the rough organisms or whether there is another carbohydrate present in both the "S" and "R" forms and not associated with virulence, similar to the "C" substance of the pneumococcus, described by Tillett, Goebel, and Avery (11) remains to be determined, since these fractions do not appear to enter into the agglutination reactions.

CONCLUSIONS

The results of cross-agglutination and agglutinin absorption experiments with the motile smooth, non-motile smooth, motile rough, and non-motile rough forms of *B. typhosus* are presented.

Cross-agglutination between these four forms is complete, save that the motile rough antigen is under certain conditions only weakly agglutinated by the antisera prepared with the non-motile forms.

Cross-absorption of the somatic agglutinogen of the four variants is complete, save that the motile smooth culture still shows granular agglutination with the anti-MS and anti-MR sera after absorption with these cultures.

A theory of the antigenic composition of the four variants of *B. typhosus* is presented, based on the results obtained in these experiments.

It would appear that, contrary to the usually accepted theory, the four variants have a common somatic agglutinogen. To explain the difference between the smooth virulent forms and the rough nonvirulent forms it has been assumed that the S forms contain a carbohydrate which is associated with virulence and which takes no part in the agglutination reaction.

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