

STUDIES ON THE DISSOCIATION OF THE HOG CHOLERA BACILLUS

II. SEROLOGICAL REACTIONS, VIRULENCE AND STABILITY OF THE VARIANT FORMS

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In a previous paper (1) a study of the dissociation of the hog cholera bacillus was presented. By using both semi-solid and solid agar media the appearance of 3 distinct variant forms was observed arising from the "normal" motile smooth (MS) strain. The 3 variant forms which appeared were designated motile rough (MR), nonmotile smooth (NS), and nonmotile rough (NR). Briefly the appearance of the colonies of the 4 forms which grew deeply in the semi-solid medium was as follows: (a) MS,—large homogenous colonies, (b) MR,—smaller irregular colonies, (c) NS,—minute compact colonies and (d) NR,—minute compact colonies. To differentiate the latter 2 forms macroscopically, it was necessary to observe the colonies as they grew on the surface of agar plates. Under this condition, the smooth surfaces of the NS colonies were distinguished from the rough surfaces of the NR colonies.

The bacteria from the S colonies, both motile (MS) and nonmotile (NS), grew diffusely when cultivated in broth and remained in diffuse suspension when placed in saline solutions. Bacteria from the R colonies, both motile (MR) and nonmotile (NR), grew in clumps in broth media and when suspended in salt solutions clumped and precipitated in flakes. It was also observed that bacilli from both S forms of colonies were more resistant to the bactericidal action of phenol and of normal rabbit serum than the organisms derived from the R colonies.

In the present report further differential tests were applied to

the 4 forms of the hog cholera bacillus. A study of the serological reactions, of the virulence and of the stability of the 4 forms was made.

Serological Reactions

Reference was previously made (1) to a schematic diagram by White (2) representing the dissociant forms of the paratyphoid group of bacteria. The probability that the flagellar and somatic antigens were identical with the H and O antigens respectively was

TABLE I
Theoretical Agglutination of the Variant Forms of Hog Cholera Bacilli Assuming that MS = OH, MR = ØH, NS = O and NR = Ø

Antigen	Immune sera			
	Anti-MS (OH)	Anti-MR (ØH)	Anti-NS (O)	Anti-NR (Ø)
MS (OH)	+	+	+	-
MR (ØH)	+	+	-	+
NS (O)	+	-	+	-
NR (Ø)	-	+	-	+

+ = agglutination.

- = no agglutination.

O = smooth, heat stable, granular, somatic antigen complex.

H = heat labile, floccular, flagellar antigen complex.

Ø = rough, heat stable, granular, somatic antigen complex.

also remarked. Under this assumption the 4 forms of hog cholera bacillus, MS, MR, NS and NR may be represented by the symbols OH, ØH, O and Ø respectively (Ø = the rough form of O). Accordingly, the theoretical serological reactions of the various forms are shown in Table I. A study of the actual serological reactions was then made.

Methods

Preparation of Sera.—For the preparation of the immune sera, bacteria from agar slant cultures were suspended in salt solution and killed at 56°C. Rabbits were then immunized by injecting suspensions of the 4 forms of bacilli intravenously 6 or 7 times at 5 to 7 days intervals. A week after the last injection the animals were bled to death and the serum was obtained.

Sera were absorbed with heavy suspensions of living bacteria in 0.1 per cent salt solution (stronger solution of salt caused spontaneous clumping of the R forms and was not used). The mixtures were incubated at 37°C. for 2 hours, placed in the ice box for 24 hours and finally centrifuged to regain the diluted serum. The final dilution of the sera was 1:20.

Agglutination Tests.—The bacteria to be tested were derived from agar slant cultures and were suspended in 0.1 per cent salt solution. Dilutions of sera in 0.1 per cent salt solution, varying from 1:40 to 1:10240, were made to which the bacterial suspensions were added. Suspensions of bacteria in 0.1 per cent salt

TABLE II
Agglutination Reactions Actually Observed

Culture tested	Agglutination titer			
	Anti-MS serum	Anti-MR serum	Anti-NS serum	Anti-NR serum
MS	5120 F	5120 F	640 G	—
MR	5120 F	5120 F	160 ?	160 G
NS	640 G	—	640 G	—
NR	—	160 G	—	160 G

F = floccular.

G = granular.

— = no agglutination.

? = differing from theoretical result.

solution served as control tests. The tubes were then incubated at 50°C. for 2 hours. Readings were made at the end of this time and again after the tubes had been in the ice box for 24 hours.

The results of the agglutination tests are shown in Table II. The figures represent the highest dilution in which macroscopic agglutination occurred. The letter F indicates a floccular type of precipitation and G indicates granular precipitation.

It is evident that the actual agglutinations represented in Table II correspond closely to the theoretical reactions predicted in Table I. An exception is noted by the agglutination of MR bacteria in a relatively low dilution of anti-NS serum. The reason for this is not clear and is at present unexplained. It is possible that the anti-NS serum contained some H agglutinins which was indicated by the fact that after the MR bacilli were heated to 100°C. for

30 minutes to destroy the H antigen, no agglutination in anti-NS serum occurred.

The titer of the anti-NR serum was low and attempts to produce a stronger anti-serum were not successful.

Different types of agglutination were observed as indicated in the table. Bacteria of the MS and MR forms when agglutinated by either anti-MS or anti-MR sera promptly formed large, loose, floccular clumps, due to the presence of the H antigen and antibody. MS bacilli in anti-NS serum and MR bacilli in anti-NR serum

TABLE III
Results of Absorption Tests

Bacterial suspensions	Anti-MS (OH) serum			Anti-MR (ØH) serum			Anti-NS (O) serum			Anti-NR (Ø) serum	
	Unabsorbed	Absorbed with MR bacilli	Absorbed with NS bacilli	Unabsorbed	Absorbed with MS bacilli	Absorbed with NR bacilli	Unabsorbed	Absorbed with MS bacilli	Absorbed with MR bacilli	Unabsorbed	Absorbed with MR bacilli
MS (OH)	5120 F	640 G	2560 F	5120 F	—	5120 F	640 G	—	640 G		
MR (ØH)	5120 F	—	2560 F	5120 F	160 G	5120 F	160 ?	—	—	160 G	—
NS (O)	640 G	640 G	—				640 G	—	640 G		
NR (Ø)				160 G	160 G	—				160 G	—

The figures represent the highest dilution in which macroscopic agglutination occurred.

F = floccular agglutination.

G = granular agglutination.

? = differing from theoretical result.

slowly formed into hard, small, granule-like clumps. Bacteria of the NS and NR forms formed similar small granular clumps whenever agglutinated by any of the 4 anti-sera, due to the presence of the O or Ø antigen and O or Ø antibody.

Absorption Tests.—The results of absorption tests which are given in Table III confirmed the results obtained by the ordinary agglutination reaction. The floccular H agglutinin was absorbed from the anti-MS (OH) serum by the MR (ØH) bacilli, but the granular (O) agglutinin remained intact. MR (ØH) bacilli absorbed the

granular (\emptyset) antigen from the anti-NR (\emptyset) serum. Anti-NS (O) serum was unaffected by MR (\emptyset H) bacilli.

Similarly the MS (OH) bacteria exhausted the floccular (H) agglutinin from the anti-MR (\emptyset H) serum as well as the granular (O) agglutinin from the anti-NS (O) serum, since these bacilli possess both O and H antigens. The granular (\emptyset) agglutinin in the anti-MR (\emptyset H) serum was, of course, unaffected.

Lastly NS (O) bacteria had no influence on the H agglutinin in anti-MS (OH) serum and the NR (\emptyset) organisms absorbed the

TABLE IV
Virulence of the Four Forms of Hog Cholera Bacilli for Hamsters (Chinese Field Mice)

Culture tested	Dose, cc.	No. used	No. died	Mortality
MS	0.1	5	5	100%
	0.001	10	10	100%
	0.000001	5	4	80%
MR	0.1	5	0	0
	0.1	10	0	0
NS	0.1	5	5	100%
	0.001	10	5	50%
NR	0.1	5	0 (abscess)	0
	0.1	10	0 (abscess)	0

Animals were observed for 30 days.

granular (\emptyset) agglutinins from the anti-MR (\emptyset H) serum but left its H agglutinin intact.

It should be mentioned that for the various serological tests only typically reacting bacteria were used. Indefinite, intermediate, or confusing forms which reacted atypically were frequently encountered and were discarded.

Virulence

Besides the differences in the serological reactions of the 4 forms of the hog cholera bacillus, striking differences in virulence for hamsters (Chinese field mice) and rabbits were observed. Varying

amounts of 24 hour old broth cultures of the 4 forms of bacilli were injected subcutaneously into hamsters. The results of the experiment are given in Table IV.

From the table it appears that in general, as in other bacterial species, the S forms were virulent and the R forms were not. The virulence of the MS form seems to be somewhat greater than that of the NS form. The NR form, however, can probably not be considered to be entirely avirulent. Hamsters receiving 0.1 cc. doses of the NR form subcutaneously occasionally died and abscesses usually developed at the site of inoculation in those that survived. The MR form on the other hand produced no gross pathological changes. Both the MR and the NR forms were frequently virulent for hamsters when injected in large doses (0.5 cc.) intraperitoneally.

Rabbits, when inoculated with the 4 forms of bacilli, reacted in a similar manner. The MS form caused death in 7 days after the subcutaneous inoculation of 0.000001 cc. of broth culture. Of 2 rabbits inoculated with 0.1 cc. of an NS culture, one died after 9 days and the other recovered after a period of fever lasting about 3 weeks. Both NR and MR organisms failed to kill rabbits even in 0.1 cc. doses, but the animals receiving NR bacilli, as in the preceding experiment, developed fever (40°C.) and appeared ill for several days but recovered.

Cultures were made from the spleen of all animals which succumbed during the course of the virulence tests. The same forms of variant bacilli as those used for inoculation were recovered.

Studies on the Stability or Reversibility of the Variant Forms

Reversion in Broth.—Attempts were made to induce reversion from one form to another. The 3 variant forms were repeatedly grown in meat infusion broth. Transfers were made twice daily for 32 days. At intervals during this procedure the cultures were plated in semi-solid media and on the surface of agar plates, and examined. The bacteria were also tested for motility and for clumping in salt solution.

During the experiment, the NR and the NS forms invariably reproduced only their respective kinds. No reversion occurred. However, after several transfers of the MR form, many MS colonies

of the original "normal" strain appeared among the MR colonies. Certain MR forms therefore reverted to the original MS form.

Effect of Animal Passage.—Broth cultures of the 3 variant forms were injected intraperitoneally into hamsters. Large doses of MR and NR strains were virulent when injected intraperitoneally instead of subcutaneously. The 3 strains were passed through 15 mice by intraperitoneal injection of peritoneal washings from mice killed by the bacilli. At intervals during this procedure the bacilli were plated in semi-solid medium and on solid agar, and the colonies were examined.

The NS and NR forms were unaffected by mouse passage and did not revert to any other form. The MR form, on the other hand, showed a tendency to revert to the MS form. After the 6th passage a few MS colonies appeared among many MR colonies. During subsequent (15) passages the proportion of MS to MR colonies gradually increased.

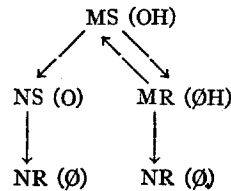
Effect of Growth in Immune Serum.—Since other bacteria have been found to revert from R to the S form during growth in anti-R sera, similar trials were made with hog cholera bacilli. The 3 variant forms NS, MR and NR were transferred every other day in broth containing 10 per cent of NS, MR and NR anti-sera respectively for 2 weeks. The cultures were plated and examined at intervals. The NS form showed further dissociation into the NR form. NR bacilli also appeared among the MR forms during the transfers. The NR forms arising from the MR form were identical with the NR forms which were derived from the NS bacilli. The NR form remained unchanged. It appeared from this test that growth in anti-R sera did not cause a reversion of the NR or MR forms to the original form but did cause further dissociation of the MR and NS organisms.

SUMMARY

The experiments recorded in the present paper confirm the existence of 4 forms of the hog cholera bacillus described in a previous paper (1), namely the "normal" type strain MS and its 3 variants, MR, NS and NR. Serological evidence is also presented to show that the symbols MS, MR, NS and NR represent the similar

conceptions of previous investigators who have used the letters OH, O, ØH and Ø respectively to designate variant forms of other organisms. It has been shown that the S form of the hog cholera bacillus, as the S forms of other bacteria, is more virulent for laboratory animals than the R forms.

In regard to the reversibility of one form to another it was found that by transfer in broth or by passage through mice the MR form showed a tendency to revert to the original MS form. Transfer in broth and animal passage, however, failed to induce any variations in the NR or NS forms. Growth in homologous immune sera did not cause reversion to original forms but in fact provoked further dissociation of the MR to the NR form and also of the NS to the NR form. The MR form may revert to the original normal MS form or may dissociate further into the NR form and is, therefore, the most unstable of the variants. Further attempts to induce changes in the other variants were not made. The dissociation as observed may be represented thus:



REFERENCES

1. Li, C. P., *J. Exp. Med.*, 1929, 50, 245.
2. White, P. B., *Med. Res. Coun., Special Report Series*, 1926, No. 103.