THE NON-IDENTITY OF AGGLUTININS ACTING UPON THE FLAGELLA AND UPON THE BODY OF BACTERIA.[1]

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The relation of flagella to agglutination has received more or less attention since Malvoz's experiments published in 1897.[2] He found that typhoid bacilli retained on the Chamberland filter after prolonged washing no longer became agglutinated by typhoid serum or by certain chemical substances such as formalin and safranin. The bacilli acted on were no longer motile and cilia were not demonstrable. The subject was taken up by Dineur,[3] who, after a painstaking series of experiments with several cultures of the typhoid bacillus, came to the conclusion that the cilia are the primary agents in the process and that the bacilli become attached to one another by means of these cilia. He goes over Malvoz's experiments and finds that the agglutinability is largely lost after processes of manipulation which injure or tear off the cilia.

H. C. Ernst and Robey[4] have gone over the experiments of Dineur, but they conclude that the "agglutinating property does not lie in and is in no way connected with the flagella of the bacteria concerned." Nicolle and Trenel[5] have recently reported observations bearing upon the same subject. They describe races of typhoid bacilli from water which failed at first to respond to typhoid agglutinin, but did so later after cultivation. They note a parallelism between motility and agglutinability. They make a distinction between

[1] Read at the Third Annual Meeting of the American Association of Pathologists and Bacteriologists, Washington, D.C., May, 1903.

[2] Annal. de l'Inst. Pasteur, 1897, no. 6.

[3] Bulletin de l'Académie Royale de Médecine de Belgique, 1898, iv. series, Vol. xii, p. 705.

[4] Trans. Congress Amer. Phys. and Surgeons, 1900, p. 26.

[5] Annal. de l'Institut. Pasteur, 1902, xvi, p. 562.

" aptitude agglutinative " and " fonction agglutinogène," and infer from their own experiments that a bacillus which has been made non-motile at 42° C. does not produce agglutinin in animals. In view of the results stated by us below, that it takes ten to twenty times the amount of immune serum producing agglutination of motile bacilli to produce agglutination in non-motile forms, it is highly probable that they did not treat their animals long enough to produce the necessary agglutinin. This explanation is strengthened by the fact that the amount of culture material injected by them is relatively small. These authors are inclined to ascribe to the cilia " le rôle capital dans le phénomène de l'agglutination."

Defalle [6] deals with the same subject with special reference to the capsules of the Friedländer and allied bacilli. He places great importance upon flagella in agglutination, but adduces no new facts.

The difficulty which is encountered at once in applying the theory that the cilia or flagella of bacteria are the chief actors in the process is that bacteria which possess no cilia are clumped in immune sera, but the agglutination appears only in dilutions much more concentrated than are necessary for the clumping of motile bacteria. The apparent contradiction between the conclusions of Malvoz and his followers and the agglutinability of non-motile forms is cleared up by the following observations which demonstrate the existence of flagellar as well as body agglutinins distinct from each other.

The following experiments were not made at the outset to determine the relation of flagella to the phenomenon of agglutination, but the facts to be reported were discovered quite accidentally in the course of a study of bacilli belonging to the hog-cholera group, with special reference to their agglutinative relationship. This study is published in the Journal of Medical Research for 1903, IX, p. 270, and for all details of the cultures employed and the methods used

[6] Annal. de l'Institut Pasteur, 1902, xvi, 595.

we must refer the reader to that publication.[1] One of the cultures included in that group was considered of sufficient importance to deserve special consideration. This was described several years ago by one of us,[2] and it has been under observation more or less ever since.

It is well known that hog-cholera bacilli are actively motile. Their motility is much more pronounced than that of *B. coli*, for it may be observed in any culture medium and in cultures many days old. Moreover, the motility is general, nearly all bacilli sharing in it, and it does not decline materially as the period of cultivation becomes longer. The culture above referred to differs from the defined type α in that the bacilli are non-motile. In all other respects it is identical with the motile type. Motion has never been seen during the years of use in this laboratory.

When the studies on the agglutinative characters of the hog-cholera group were begun by one of us in 1898, the intimate relation between the motile and the non-motile type was soon made evident, and mentioned in the article quoted. During the past year a more detailed investigation of this relationship was made, which has revealed certain important facts destined to explain hitherto controversial views, and to illustrate the complex nature of agglutination phenomena already suspected by many who have devoted time to this subject.

The action of the serum of rabbits and guinea-pigs immunized with the non-motile bacillus upon this bacillus differs in several particulars from the action of serum produced by motile bacilli on the latter.

1. The clumping appears at least several hours later.

2. The clumps appear in the form of a uniform precipitate of compact granules, at first excessively small and later

[1] As a supplementary note to the observations reported in that paper, it should be stated that the members of the group there studied act alike upon mannite, and in the same way as they act upon dextrose. This medium does not, therefore, distinguish between the individuals. It should also be stated that a culture of *B. icteroides* recently received through the kindness of Dr. Flexner, directly from Dr. Sanarelli shows the same agglutinative affinities toward the hog-cholera bacillus α as does the culture from Dr. Reed, used in the published tests.

[2] Centralblatt für Bakteriologie, 1899, xxv, 241.

growing larger, if the serum is of the proper strength. Loose flocculi so common in the clumping of motile bacilli are absent.

3. The agglutination appears only after a much higher degree of immunization of the animal yielding the serum than is necessary with the motile bacilli.

These observations lead to the inference that the agglutinin affecting non-motile bacilli is produced with more difficulty and acts more slowly, so far as mere optical tests go, than the agglutinins produced by motile bacilli. Furthermore, a study of the clumping process in the presence of motile bacilli with serum prepared with such bacilli indicated that the flagella were chiefly acted upon. The clumps began as loose collections of bacilli, not touching one another, but separated by narrow spaces. The entire mass floats as one, but the bonds that connect the individuals are not in sight. The clumps of the non-motile bacilli are compact. The bacilli are not separated by any appreciable space.

Those who have given attention to the clumping of motile and non-motile bacteria in the hanging drop, with immersion lenses, have without doubt noticed these differences, and so far the phenomena may be considered well known. Only by a study of the reciprocal action of immune serum upon these closely related motile and non-motile bacilli were certain new facts elicited.

Rabbits were immunized by repeated injections of living and dead cultures of the motile hog-cholera bacillus α from different sources. The serum, after four or five weeks of treatment, would as a rule show distinct clumping with a hand lens in dilutions above 1:10,000; sometimes in dilutions above 1:30,000. These figures represent limits; that is, dilutions of one-half the strength given would not show any change in the tubes after two to four hours. When the non-motile hog-cholera bacilli were exposed to the same serum, they would clump only in dilutions up to 1:500. The same was true of the serum prepared with *B. icteroides* which, as stated in the preceding paper, acted precisely like hog cholera α in all agglutination tests.

TABLE I.

Culture used for immunization.	Number of rabbit.	Agglutination towards its own bacillus.	Non-motile hog-cholera bacillus.
Icteroides (orig.)	128	8,000	500
Hog cholera, Md.....	93	32,000	500
Hog cholera, Mass.	143	16,000	500
Swine dysentery ∝	158	2,000	< 20
Guinea-pig disease ∝	157	4,000	< 20

NOTE. — For the history of the cultures and the immunization of the rabbits see Journ. Med. Research, 1903, ix, p. 270.

It might be claimed that the figures above given do not establish any close relation between the motile and non-motile type of *B. cholerœ suis*, but the objection is removed when we consider that the serum of none of the other more distantly related bacilli acted on the non-motile bacillus in dilutions of 1 : 20, or even 1 : 10, and that the relation given holds for hog-cholera ∝ from various sources, as well as for its congener, *B. icteroides*.

It would seem, then, that a serum agglutinating motile bacilli acts upon non-motile bacilli of the same biological and pathogenic characters, but in dilutions at least twenty times as concentrated.

The immediate inference which might be drawn is that the agglutinins which readily act upon the flagella of bacteria are the same as those acting upon the bodies of bacteria, but they must be much more concentrated in order to produce any visible effect upon the envelope of the bacteria. If this were true, a serum prepared with the non-motile bacilli and agglutinating the latter at 1 : 500 should attack the flagella of the motile forms much more readily and produce agglutination in higher dilutions, *i.e.*, up to 1 : 10,000 and above.

But the experiment did not support any such inference. The serum produced with the non-motile bacillus acted upon

hog-cholera *a* and *B. icteroides* very much as upon the non-motile bacillus itself, as shown in table II. In general, the clumping of the motile bacillus required dilutions a trifle more concentrated, while the serum failed to act upon other bacilli of the same group in dilutions of 1:10 with two isolated exceptions (guinea-pig disease *a*).

TABLE II.

DESIGNATION OF ANIMAL.	Dose and number of injections of living culture of non-motile hog-cholera bacillus.	Method of Testing.	Bacillus Coli VI.	Typhoid V.	Icteroides (Sanarelli.)	Gallstone bacillus.	Hog cholera non-motile.	Hog cholera Maryland.	Swine dysentery α	Spermophile.	Guinea-pig disease α	Other cultures.
Guinea-pig, 2302	0.5 cc. of 48-hour bouillon culture.	Hanging drop under microscope.	<10	10	200	<10	**500**	200	<10	<10	<10	Hog cholera, Arkansas —500. " " Mass. — 200. " " Minnesota—500. " " Nebraska — 500. Gall bladder — <10.
Guinea-pig, 2341	0.5 cc. 48-hour bouillon culture. 1 cc. 24-hour culture (2 injections).	Hanging drop under microscope.	<10	10	200	<10	**200**	100	10	<10	<10	Hog cholera, Arkansas — 200. Gall bladder — <10.
Guinea-pig, 2547	Bouillon suspension of 1 agar slant twice.	In test tubes.	—	<10	100	<10	**100**	50	10	<10	50	Bacillus Coli IX. — <10. " " X. — <10. Swine dysentery β — <20. Guinea-pig disease β — 20.
Rabbit, 139	Bouillon suspension of 1 agar slant twice.	In test tubes.	—	<10	50	<10	**100**	20	10	<10	20	Bacillus Coli IX. — <10. " " X. — <10. Swine dysentery β — <20. Guinea-pig disease β — 10.
Rabbit, 149	1 cc. 96-hour bouillon culture. 1 cc. 48-hour culture 1.5 cc. 120 hour culture (3 injections).	In test tubes.	<10	<10	100	<10	**400**	100	<10	<10	<10	Icteroides (Santiago) — 200. Swine dysentery β — <10. Shiga — <10. Hog cholera, Mass. — 100. " " Nebraska— 400. " " Arkansas — 200.
Rabbit, 153	0.75 cc. 6-day bouillon culture. 1 cc. 8-day culture (2 injections).	In test tubes.	<10	<10	200	<10	**200**	200	<10	<10	<10	Hog cholera, Arkansas —200. " " Mass. — 200. Bacillus Coli X. — <10. " " XII. — 40.

The explanation of this behavior of the serum from the non-motile bacillus became clear on further study. Above all, the phenomenon of clumping itself was instructive. In place of the large, fluffy precipitate so frequently seen with motile bacilli, only a uniformly fine compact granular precipitate appeared, wholly similar to that in the tubes of the non-motile bacillus. Later the small clumps gathered into larger ones and subsided. The suspicion was at once aroused that the "non-motile" serum contained agglutinins for the bodies of the bacilli and not for their flagella, and that the process of agglutination affecting the bodies was qualitatively and not simply quantitatively different from that of the flagella. Further studies, especially of the clumps, failed to show anything to contradict this theory. The clumps of motile bacilli produced by "non-motile" serum do not begin with the regular spacing of bacilli, but they appear more or less compact from the start. The bacilli form compact bands which attach themselves to one another and thus form an interlacing mass when a number of smaller clumps have united. The behavior of the flagella throughout is of interest. The small clumps move about actively. Signs of paralysis are absent. When the smaller masses have drifted together, the larger clumps show throughout a protean change of outline due to the activity of the uninjured flagella.[1]

In order to determine the exact relation existing between the flagellar and the body agglutinin, saturation experiments were tried to remove one or the other agglutinin. It should be borne in mind at the outset that in the non-motile serum only one agglutinin is postulated by the theory, while in the motile serum both flagellar and body agglutinins may be

[1] The action of the body agglutinin upon motile bacilli is frequently seen when serum of sufficient concentration is used. In such cases, the flagellar agglutinin being present in abundance, relatively large flocculi are formed, which subside and unite into a loose, snowy mass in the bottom of the tube. This fluffy deposit, if left undisturbed, begins to shrink, and within twenty-four hours has contracted probably on account of the action of the body agglutinin into a relatively minute mass. After the clumps which form at first have subsided, a fine, powdery, or granular precipitate is frequently seen. This is probably the result of the body agglutinin acting on the bacilli deprived of flagella.

looked for, since motile bacilli stimulate the formation of both.

1. Serum[1] from a rabbit (143) treated with motile bacilli (hog cholera, Mass.) was diluted 1 to 50. Into this dilution non-motile bacilli were put from an agar slant until the fluid became quite turbid. It was placed in the incubator for four hours, centrifugalized, and filtered through filter paper, and a clear fluid was obtained. This showed that the serum had not been fully saturated, otherwise we should look for a faint cloudiness remaining. This treated serum was compared with an untreated serum in different dilutions toward the two bacilli used for immunization, also toward an old hog-cholera culture and *B. icteroides* (Sanarelli). The method employed is the same as that described in a foregoing paper, *i.e.*, small test tubes were used, and the fluid examined with the naked eye, a hand lens, and occasionally in the hanging drop, with a $\frac{1}{12}$ oil immersion objective.

The result showed a diminution of the body agglutinin toward the non-motile bacillus from 1 : 500 to 1 : 100. There was no loss of flagellar agglutinin, for the series of tubes containing treated and untreated serum presented the same appearance. The precipitate was very fluffy, and snow-like in character.

2. This experiment was repeated with serum from a rabbit treated with hog cholera, Md. The serum used in a dilution of 1 : 50 was supersaturated with the non-motile bacillus, placed in the incubator for several hours, and then in the refrigerator over night. After repeated filtration through paper a faint cloudiness still remained, showing the absence of any free body agglutinin. With this serum dilutions were made and similar dilutions of untreated serum.

The untreated "motile" serum clumped the non-motile bacillus in dilutions up to two hundred. The treated serum did not, having been supersaturated with it. Both treated and untreated serum clumped the motile bacillus in dilutions up to 1 : 20,000.

[1] For details of immunization, see Journal Medical Research, 1903, Vol. ix, p. 270.

3. Serum from an immune rabbit (153) which had received two doses of living bacilli of the non-motile variety was found to have agglutinin in the following concentration:

Non-motile bacillus, 1:200 . . trace at 1:500
Hog cholera, Md., 1:200 . . " "
Hog cholera, Ark., 1:200.

This serum, in a dilution of 1:20, was supersaturated with motile hog cholera, Md., from agar slants, placed in the incubator for several hours and in the cold over night. Next morning the fluid was concentrifugalized, but a faint cloudiness remained, showing an excess of bacilli in the presence of agglutinin.

The faintly clouded, treated serum was used for dilutions, and parallel dilutions were made with untreated serum.

In the treated serum the agglutinin toward the non-motile bacillus had fallen from 1:500 (trace) to 1:40 (trace). In the 1:40 dilution a dense granular precipitate still formed with the motile bacillus (Md.) just visible with the hand lens. This shows that even the supersaturation had left some agglutinin behind.

We may infer, therefore, that while no appreciable effect is produced upon serum prepared with the motile hog-cholera bacillus and saturated with the non-motile bacillus so far as the clumping of motile bacilli is concerned (flagellar agglutinin), the removal of the body agglutinin from the non-motile serum by saturation with motile bacilli affects the agglutinin of the non-motile bacillus, and thus indicates identity of the two body agglutinins.

Another experiment which suggested itself is the removal of the flagellar agglutinin by motile bacilli and a testing of the influence of this removal on the body agglutinin. The difficulty encountered here is the absorption of both agglutinins at the same time. The experiment was, however, made by adding to "motile serum" (hog cholera, Mass.) its own bacilli, incubating for an hour and then removing the bacilli by filtration. The filtrate was found to have lost decidedly of both body and flagellar agglutinins, in spite of the short period of incubation. The proportion of loss seems to

have been nearly the same for both motile and non-motile bacilli.

The foregoing experiments are sufficiently complete at present to warrant the conclusion that the agglutinins for the flagella and for the body of bacilli, at least so far as the large group of pathogenic colon derivatives is concerned, are distinct, not mutually interacting substances. It is obvious that but for the existence of these two varieties of bacilli (one motile and the other not) the demonstration could not have been successfully made. With the new facts in view it may now be possible to carry out more effectually Malvoz's experiments of depriving motile bacilli of cilia and demonstrating upon them the presence of a second agglutinin.

The existence of agglutinins for at least two different organs of bacilli will obviously strengthen the theory that agglutinins act directly upon the structures rather than indirectly through precipitants. The theory of Gruber-Durham that the agglutinins produce a stickiness of the parts acted upon seems on the whole the simplest explanation of the facts related. Similarly, many anomalies of agglutination depending upon partial loss of motility of cultures and upon the irregularities in the clumping of cultures of *B. coli* will be cleared up by the knowledge of at least two distinct agglutinins. The thread reaction, upon which controversies have been waged, will also be open to explanation, as in our experience it seems to be closely associated with the body agglutinin.

These experiments go a step farther in supporting the thesis presented in the preceding paper that the host is responsible, within certain limits, for the agglutinative characters of the bacillus parasitic in that host. These limits are the original capacity of the bacilli, *i.e.*, the possession of certain receptors and the degree of parasitism attained. Invasion of the blood and tissues and multiplication therein represents the highest degree, and bacteria of the same species, having acquired this power toward the same host, usually agglutinate alike. The ancestry of the motile and the non-motile races of the hog-cholera bacillus will remain in doubt,

and any speculation concerning it can do little more than stimulate efforts to elucidate it. Some years ago, in the paper referred to, one of us presented the hypothesis that the motile races are derivatives of B. coli or its progenitor, and the non-motile race a derivative of B. lactis aërogenes.

CONCLUSIONS.

1. The non-motile race and the motile races of the hog-cholera bacillus manifest a close affinity towards one another in the presence of immune agglutinins. This is also true of B. icteroides, Sanarelli.

2. This affinity enables us to differentiate the agglutinins of motile bacilli into flagellar and body agglutinins.

3. The agglutinin acting upon the bodies of the non-motile hog-cholera bacillus is identical with that acting upon the bodies of the motile race or species, but different from that acting upon the flagella.

4. The flagella agglutinins are much more easily demonstrated in immune sera. In the cultures studied the presence of the former was manifested in dilutions over twenty times greater than in those in which body agglutinins became visible.

5. In order to obtain body agglutinins, a much higher degree of immunity must be induced.

6. The assumption of two agglutinins as defined above will probably serve to clear up various apparent discrepancies in agglutination tests and explain the so-called thread reaction.