AGGLUTINABILITY OF BLOOD AND AGAR STRAINS OF THE TYPHOID BACILLUS

*STUDIES IN TYPHOID IMMUNIZATION. II**

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The growth of the typhoid bacillus on an agar-medium containing 10 per cent. defibrinated rabbit blood, which we have found of advantage in our experiments, differs in certain respects from the growth on plain agar. We have already noted that the blood-agar cultures are more abundant. A concrete example of the amount of growth produced on each medium may be drawn from certain of our vaccine preparations to be more fully described later. To produce growths of *Bacillus typhosus* for immunizing vaccines we inoculate one-quart Blake bottles containing 100 c.c. of medium with a few cubic centimeters of a twenty-four-hour bouillon-culture of the organism. In one experiment nine Blake bottles of 2 per cent. agar were inoculated with a plain bouillon-culture of *B. typhosus* No. 3; at the same time, nine Blake bottles of 10 per cent. blood-agar were inoculated with a blood-bouillon culture of *B. typhosus* No. 3b. After incubation at 37 C. (98.6 F.) for two days, the collected agar and blood-agar growths were suspended each in 220 c.c. of normal saline, filtered through cotton and precipitated with 300 c.c. of absolute alcohol. After centrifugalization, the separate precipitates were again centrifugalized and dried over sulphuric acid for two days. These dried cultures were ground and weighed with the following results: Blood-growth, 0.675 gm.; agar-growth, 0.36 gm. It will thus be seen that the growth of the organism is almost twice as abundant on 10 per cent. blood-agar as on plain agar. Not only is the growth of the blood organism more abundant, but its morphology differs distinctly from the agar-strain. When examined in hanging-drop preparations, the blood-strain of the typhoid bacillus is larger, thicker and tends to grow in chains. It remains, however, quite as motile as the agar variety.

Associated with the morphological change that the organism undergoes when grown on rabbit blood, and we believe in some way dependent on it, are certain distinct and important variations in the agglutinability of these organisms by antityphoid serum. The ultimate identification of bacilli isolated from the blood-stream of suspected typhoid-carrier rabbits

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depends on their agglutination by means of a potent antityphoid serum. Our first attempt to agglutinate such a culture (*B. typhosus* 3a), which culturally and morphologically agreed with the characteristics of *B. typhosus,* with a potent antityphoid serum from the rabbit, was a complete failure. The rabbit furnishing this serum had been immunized by repeated inoculations of killed agar-cultures of *B. typhosus* suspended in saline and its serum agglutinated the stock cultures with which we were working in dilutions of from 1:10,000 to 1:20,000. The failure of this potent diagnostic serum to agglutinate the cultures derived from the suspected carrier in dilutions of 1:100, in view of the fact that these cultures were isolated on a blood-medium, whereas they had been growing on an agar-medium before inoculation into the rabbit, led us to suspect that we might be dealing with the phenomenon described by Bordet and Sleeswijk with the whooping-cough bacillus.

Bordet and Sleeswijk,[1] it will be remembered, found that the serum of an animal that had been immunized by injections of agar-growths of the Bordet-Gengou whooping-cough bacillus would clump similar agar-strains, but had no effect on blood-culture growths of the same organism. The serum of an animal immunized by means of the blood organisms would, however, agglutinate both varieties of whooping-cough bacilli. With these observations in mind we immunized several rabbits with blood-cultures of *B. typhosus,* and compared the agglutinating properties of their serums with the serums of rabbits that had received agar-cultures of the organism.

In Table 1 are groupted the results from a series of experiments with antiserums from rabbits obtained in the manner indicated. The first three serums in the vertical columns are from rabbits that had been given injections of agar-cultures of *B. typhosus;* the other six serums are from animals that received blood-cultures (*B. typhosus* 3a).

Table 1 shows clearly the following facts: An antityphoid serum from a rabbit that has been immunized by blood-agar cultures of *B. typhosus* has the property of agglutinating indiscriminately, to the limit of its potency, both blood and agar-cultures of the organism.[2] The serum of a rabbit immunized against agar-cultures agglutinates the agar organism only. The inagglutinability of blood-cultures by antiagar serum is not due to some property acquired in the living animal, as similar results can be obtained by simple subculture for two generations of the agar-strain on blood-medium. The agglutinability may be restored by retransference to the agar-medium. Growth in bile produces the same results as growth in blood.

1. Bordet and Sleeswijk: Séro diagnostic et variabilité des microbes suivant le milieu de culture, Ann. de l'Inst. Pasteur, 1910, xxiv, 476.

2. It may be noted, however, that the agglutination of the blood-strains invariably proceeds more slowly than that of agar-cultures.

The variation in the morphological appearance of the blood-agar cultures suggests that the failure of these organisms to agglutinate might be due to some physical difference acquired by growth on this medium. It is known that the agglutinability of members of the *mucosus capsulatus* group of bacteria, for instance, is dependent on the absence of a capsule.[3]

Two recent articles seem to prove that the typhoid bacillus may be capsulated either under exceptional conditions, or perhaps as a rule, provided proper strains are used. Kuhnemann[4] found capsules could be

TABLE 1.—LIMITS OF AGGLUTINATION OF AGAR AND BLOOD-AGAR STRAINS OF B. TYPHOSUS WITH SERUMS DERIVED FROM RABBITS BY IMMUNIZATION WITH AGAR OR WITH BLOOD-AGAR CULTURES

Strains of *B. Typhosus*	Antiagar Culture Rabbits			Antiblood Culture Rabbits					
	4	5	6	19	39	42	50	51	52
Agar 5	20,000	10,000	2,000	2,000	4,000	2,000	2,000	500	8,000
Agar 3	10,000	.....	.....	.....	.....	.....	.....	1,000	1,000
Blood 3a	100*	100*	100*	2,000	.....	2,000	1,000	1,000	500
Blood 3b	100*	.....	.....	.....	1,000	.....	.....	.....	1,000
Blood 3^2	100*	.....	.....	.....	1,000	.....	.....	.....	.....
Blood 3^3	100*	.....	.....	.....	.....	.....	.....	.....	1,000
Blood 5^3	100*	.....	.....	.....	.....	.....	.....	.....	2,000
Bile 3	100*	.....	.....	.....	1,000	.....	.....	.....	.....
Bile 5	100*	.....	.....	.....	1,000	.....	.....	.....	.....

* Negative.

The figures indicate the highest dilutions of the serum at which a positive agglutination was obtained. The macroscopic method was uniformly employed, each tube containing a dilution of the serum in 1 c.c. of salt solution plus a uniform amount of the organism; in the case of the suspended cultures from blood or agar, this was obtained by standardizing the cultures to a uniform turbidity and using 0.1 c.c. The final result was read after the tubes had stood for two hours at room temperature and then over night in the ice-chamber. No doubtful or partial results are noted and the results were controlled by adding each suspension to salt solution (1 c.c.). The "bouillon-bile" cultures consisted in adding 1 c.c. of a forty-eight-hour culture on this medium to the serum dilution. In the left hand column are the cultures of *B. typhosus* tested in relation to their origin and the mediums on which they had been grown before being tested. "Agar 3" and "Agar 5" refer to the original strains employed. "Blood 3a" and "3b" indicate the Culture 3 passed through one or two carrier rabbits respectively; "Blood 3^2," "3^3," "5"" indicates that the respective cultures had been simply transplanted from agar on the blood-medium for two or three generations with twenty-four hours between transplants. The bile cultures of five and three were subcultured twice on bile-bouillon before being tested.

3. Beham: Die agglutinatorischen Eigenschaften der Kapselbazillen, etc., Centralb. f. Bakteriol. 1912, Abt. I, Orig., lxvi, 110; Fitzgerald: Agglutination of Encapsulated Bacteria, Proc. Soc. Exper. Biol. and Med., 1912, x, 52.

4. Kuhnemann: Ueber Kapselbildung beim Typhusbazillen, Centralbl. f. Bakteriol., 1911, lvii, 497; Zur Identifizierung des Bacillus Faecalis Alcaligenes, Centralbl. f. Bakteriol., 1911, lvii, 469.

demonstrated by his modification of Loeffler's flagellum stains on typhoid bacilli that had been treated and clumped by fresh serum of young rabbits. He is inclined to regard this capsule formation as a protective mechanism on the part of the typhoid bacillus, as he found it only in such specially treated cultures. Carpano,[5] on the other hand, regards the failure hitherto to stain capsules on the typhoid bacillus as a purely technical matter, and he has succeeded in staining them on strains of the micro-organism on various culture-mediums.

Our own experiments with these stains have been convincing.[6] We had thought it might be possible to show capsules on the blood organisms with one of these stains while the corresponding agar-culture remained free, thereby indicating the correspondingly greater resistance to agglutination that is shown by the blood-strains. Such results, however, were not realized. With Kuhnemann's method the clear-cut capsules were found about all typhoid bacilli treated as he suggests with fresh young rabbit serum. We differ with him, however, in finding a considerable number of capsulated bacilli in twenty-four-hour preparations of both agar and blood-agar cultures. With Carpano's method we had no difficulty in demonstrating capsules on both the agar and blood-agar cultures. They are found surrounding each organism, clearly cut and uniformly. We have not succeeded, then, in demonstrating any structural basis for the differences in agglutinability of the agar and blood-agar strains of the typhoid bacillus.

It occurred to us that the relative inagglutinability of the blood-cultures might be due to the presence of some protective substance produced in the growth of these organisms. An attempt to prove this hypothesis by adding the supernatant extract of ground blood bacilli to agar-cultures resulted negatively; the agar-bacilli so treated were clumped as well as untreated bacilli by the antiagar-culture serum.

Absorption experiments have led hitherto to rather confusing results, the differences depending in the main on employment of more or less agglutinable strains of the bacillus. We prefer to reserve expression of opinion on this point to a later communication.

The practical interest of the relative inagglutinability of blood-strains of *B. typhosus* lies in its applicability of recently isolated strains of the micro-organism. In our preliminary communication,[7] we expressed the belief that freshly isolated typhoid bacilli which failed to respond to the characteristic serum test might respond more readily to an immune serum "obtained by immunizing animals with cultures grown on rabbit

5. Carpano: Ueber die Kapsehulle einiger Bakterien, Centralbl. f. Bakteriol., 1913, lxx, 42.

6. We are indebted to Miss Agnes Scholl for certain of these observations.

7. Gay and Claypole: Induced Variations in the Agglutinability of Bacillus Typhosus, Jour. Am. Med. Assn., 1913, lx, 1141.

(or possibly only on human) blood-agar." Such anticipation we have since been able to verify in practice.

It is a well-known fact among workers in diagnostic laboratories that cultures isolated from suspected cases of typhoid fever or from the stools of typhoid-carriers not infrequently fail at first of identification by their non-agglutinability with an antityphoid serum. Experience seems to vary as to the frequency with which such inagglutinable organisms are met. Thus Ledingham[8] says he has had little trouble in agglutinating fresh cultures from "carriers." Sawyer,[9] on the other hand, found that his eventually successful culture from a typhoid carrier on shipboard consisted in numerous colonies of organisms that biologically agreed with the characteristics of *B. typhosus,* but could not be finally identified by an agglutination test until they had been subcultured for ten to twelve generations on agar. Courmont and Rochaix[10] have studied hundreds of typhoid strains isolated from blood and stools of typhoid patients and also from dogs that had been given the dejecta of such patients. They state that many of these organisms are at first little agglutinable and do not become so until they have been subcultured in bouillon for ten to twelve generations.

Schiller[11] has found great variation in the agglutinability of suspected typhoid bacilli isolated from stools, some organisms from a given stool agglutinating readily with a strong antityphoid serum (titer 1 to 8,000), while others from the same stool failed to be clumped in a dilution of 1 to 100.

Kolle and Hetsch[12] state that very few real typhoid bacilli fail to agglutinate entirely, but that many agglutinate only feebly. Paltauf[13] states that the well-known failure of recently isolated typhoid cultures to agglutinate at best delays diagnosis and frequently leads to error.

Raubitschek and Natonek,[14] in a recent comparative study of strains of typhoid bacilli, express doubt of the absolute identity of some of their strains in view of the fact that some of them fail to agglutinate with a

8. Ledingham: The Carrier Problem in Infectious Diseases, Longmans, Green & Co., London, 1912.

9. Sawyer: A Typhoid Carrier on Shipboard, Jour. Am. Med. Assn., 1912, lviii, 1336, and personal communication.

10. Courmont and Rochaix: Technique de la détermination du bacille d'Eberth par la recherche de l'agglutination, Compt. rend. Soc. de biol., 1910, lxix, 134.

11. Schiller: Beiträge zur Typhus-Epidemiologie, Centralbl. f. Bakteriol., 1908, xlvi, 385.

12. Kolle and Hetsch: Die experimentelle Bakteriologie, etc., Urban and Schwarzenberg, Berlin, edit. 3, 1911, p. 261.

13. Paltauf: Die Agglutination, Handbuch der pathogenen Micro-organismen, Kolle and Wassermann, Ed. 2, 1912, ii, 505.

14. Raubitschek and Natonek: Ueber Unterschiede in den biologischen Eigenschaften der Typhusbacillen, Centralbl. f. Bakteriol., 1913, I Abt., orig., lxix, 241.

strong antityphoid serum. It is interesting to note that their cultures have all been recently isolated from various organs of cadavers.

We have of late had the opportunity of examining three recently isolated typhoid cultures, one of which (D) was reported as failing to agglutinate with an antityphoid serum.

TABLE 2.—AGGLUTINATION OF FRESHLY ISOLATED STRAINS OF B. TYPHOSUS IN VARIOUS GENERATIONS WITH ANTIAGAR AND ANTIBLOOD IMMUNE SERUM FROM THE RABBIT*

B. Typhosus Culture Generations on Agar	Antiagar Agglutinating Serum 5†	Antiblood Agglutinating Serum 42
Stock 5	1 : 16,000 +	1 : 2,000
A 1	1 : 1,000	1 : 500
B 2	1 : 100	1 : 2,000
C 2	1 : 10,000 +	1 : 2,000
D 2	1 : 100	1 : 500
D 5	1 : 100	1 : 2,000
D 8	1 : 1,000	1 : 2,000
D 9	1 : 1,000	1 : 2,000
D 12	1 : 2,000	1 : 2,000

* Cultures tested: (A) From Dr. J. V. Cooke, University of California Hospital. Original culture from spleen (necropsy 1337, May 24, 1913). Kept on ice without subculture from May 27 to June 12; (B) From Dr. J. V. Cooke. Cultures from blood of patient furnishing culture A taken three days before death. One transplantation. Kept on ice from May 23 to June 12; (C) From Dr. J. V. Cooke. Blood-culture from Mrs. N. taken May 13, 1913. Second transplant. Kept on ice from May 15 until test June 12; (D) From Dr. E. Foster. These cultures were tested with both an antiagar culture typhoid serum from rabbit (5) and an antiblood culture typhoid serum from rabbit (42); the least agglutinable culture was repeatedly sub-cultured at twenty-four-hour intervals on agar until it became readily agglutinable by the antiagar as well as the antiblood serum.

† The plus sign indicates that the limit of agglutination was not reached in the given experiment.

Table 2 shows that the antiagar-culture serum 5 is the more potent, agglutinating the Stock Culture 5 in a dilution of 1 to 20,000 (Table 1). The antiblood-serum agglutinates the stock organism in a dilution of 1 to 2,000 only. Of the freshly isolated cultures, one only, C in the second transplantation, reacts like a stock culture of *B. typhosus* to an antiagar or the usual diagnostic serum. Culture A reacts relatively feebly to the antiagar serum so that diagnosis might be questioned. Cultures B and D react only in a dilution of 1 to 100, and it must be remembered that they were used first when in the second generation on agar. Even in this generation their diagnostic identification as *B. typhosus* could not be accepted. But the point of most interest is that all the organisms react strongly with the absolutely weaker antiblood-culture serum. The reaction of Culture B2 is particularly striking—diagnosis negative or at best doubtful with the antiagar-serum, but strikingly positive with antiblood-

serum, 1 to 2,000. The subsequent generations of D show how the growth on agar renders the inagglutinable organism susceptible to an ordinary rabbit immune serum obtained with agar-cultures.

The conclusion seems justified that the diagnosis of suspected typhoid bacilli can be rendered immediately certain even on first isolation by the use of an immune serum obtained by treating animals with cultures of the organism grown on blood. The application of this diagnostic procedure to other organisms such as *B. dysenteriae* (particularly of the Shiga type) would be interesting.