THE RELATION OF THE CAPSULAR SUBSTANCE OF B. COLI TO ANTIBODY PRODUCTION.

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In several previous communications[1] an early mutation of certain capsulated strains of *B. coli* was described. The strains were obtained from the ileum of young calves, dying as a result of the multiplication of *B. coli* in the small intestine and the resulting general intoxication or septicemia. The original colony on agar plates sent out lateral expansions or wings much thinner than the original nucleus of the colony. The bacteria making up this secondary growth were without a capsule and about 1/20 to 1/25 the virulence of the nucleus when injected into the peritoneal cavity of guinea pigs. The bacteria of the colony nucleus were agglutinated only in very low dilutions of the serum of cows treated for months with intravenous and subcutaneous injections of living cultures and cultures heated at 62°C. The mutant was readily clumped in this serum in high dilutions. The agglutination of the (a) or original strain differed from that of the mutant or (b) strain in that a coherent mucoid disc formed in the bottom of the agglutination tube up to 1/10 dilution. In higher dilutions no clumping of any kind occurred. The serum from the cows treated with the original or parent strain protected guinea pigs receiving a surely fatal intraperitoneal dose of both original and mutant strains in doses of 0.005 to 0.01 cc. The serum of untreated cows failed to protect in doses up to 2 cc. With exceptions given below the original (a) strain has maintained its characters in agar slants kept at about 40°F., provided drying out was avoided and the transfer made from the bottom of the tube where some condensation water is always kept. The mutant

[1] Smith, T., and associates, *J. Exp. Med.*, 1927, xlvi, 123–166.

(b) likewise has not changed under these conditions. Virulence of (a) as measured by intraperitoneal injection of living bouillon cultures has fallen, since the minimum fatal dose has risen from 0.025 to 0.06 cc. during 3 years. In the same time the minimum fatal dose of (b) has risen from 0.6 cc. to 1.3 cc. For the bouillon cultures the same lot of bouillon was kept in storage and the cultures used after precisely 24 hours incubation. When a new lot of bouillon was used, the minimum fatal dose was again titrated. In all individual tests controls were introduced however. These two forms (a) and (b) derived from the same bacterium in the colony ((b) obtainable without subjecting bacteria to the Porges' or similar treatment) seemed suited for an examination into the protective and agglutinating antibodies following hyperimmunization.

Agglutination of (a) and (b) Strains.—The sera used in these trials were drawn from cows which had received subcutaneous and intravenous injections of *B. coli* at variable, usually 10 day intervals. *Cow A* received injections of *living B. coli* (a) from November, 1925, to July, 1927. During this period there were several gaps of 3 or 4 months during which treatment was omitted. *Cow B* received injections of *B. coli* (a) heated at 62°C., from September, 1926, to July, 1927. *Cow C* was treated in the same manner but with heated cultures of the mutant (b). In all 17 injections were given to *C*, partly intravenous, partly subcutaneous, extending from Dec. 2 to March 30, 1928. *Cow D* represents sera from various untreated cows. The blood was drawn at different stages of the immunization process into large jars, allowed to clot, and the serum drawn off, passed through Berkefeld filters and stored in full bottles at about 40°F. No appreciable degeneration of the antibody content occurred after months of storage. Agglutinins towards the mutant (b) occur in sera of untreated cows. Clumping may be complete in a 1/80 dilution.

During the immunization of the cows agglutinins towards the original strain (a) failed to appear even in 1/10 dilution until at the end of the treatment when in a 1/10 dilution of cow sera A and B a compact, coherent, mucoid disc formed in the bottom of the agglutination tube. In the 1/20 dilution indications of clumping were noted under the microscope in chain formation of a certain number of bacilli. On the other hand the titer of agglutinins towards the (b) form continued to rise from the start. Towards the end of the treatment agglutination was still recognized under the microscope at a dilution of 1/10,240.

The Thread Reaction.—To determine the influence of agglutinins on bacteria during multiplication bouillon plus immune serum was inoculated and incubated. This method was used first by Ledoux-

Lebard[2] and the following year by Pfaundler[3] and called by him the thread reaction. With this method the clumping of (a) or disc formation was observed at a somewhat earlier period of the immunization process than when the usual method was employed. Several points need consideration when this method is used. In the first place bacteria are exposed to the agglutinins during multiplication. In the second place, the few bacteria introduced at the start are exposed to the full strength of the agglutinin. This gradually diminishes as the bacteria multiply and a point is reached when the agglutinin is entirely used up and the bacteria continue multiplying without clumping.

It was thought that by incubating the bacteria in various dilutions of serum in bouillon in the hanging drop some differences between (a) and (b) in the mode of agglutination might be noted. Thus far this has not been realized. Both (a) and (b) in suitably concentrated immune serum-bouillon grow in the form of interlacing chains of bacilli, each colony forming a distinct circular lacework of chains. Only when the agglutinins are exhausted is this form concealed and modified by diffuse growth. It was thought that this method of allowing growth in serum-bouillon might be useful in the absorption of agglutinins and the following experiments were carried out.

Direct and Reciprocal Absorption of (a) and (b) Agglutinogen in (a) Cow Serum.—When a 1/10 dilution of serum in bouillon was inoculated with a loop of Strain (a) and (b) and incubated the following phenomena were observed after 24 hours.

Strain (a) in Immune Serum-Bouillon.—The fluid is nearly clear. In the bottom is a compact disc-like mass, not disrupted by shaking. The fluid is considerably clouded by the shaking.

Strain (a) in Normal Serum-Bouillon.—Heavy clouding without any coherent deposit.

Strain (b) in Immune Serum-Bouillon.—Growth only in bottom of tube. Fluid clear. When shaken, the fluid becomes heavily clouded with flakes. No disc-like coherent mass. The upturned growth soon subsides.

Strain (b) in Normal Serum-Bouillon.—After 24 hours general

[2] Ledoux-Lebard, *Ann. Inst. Pasteur*, 1897, xi, 909.

[3] Pfaundler, M., *Centr. Bakt., 1. Abt.*, 1898, xxiii, 9, 71, 131.

clouding with a lumpy deposit, easily broken up into smaller lumps, and general cloudiness.

In the course of the following 3 or 4 days there was increasing cloudiness in the immune serum-bouillon tube of (a). The gelatinous disc did not disappear. In the (b) tube the fluid remained clear. All growth took place in the bottom. Evidently in (a) the agglutinins were quickly used up and multiplication went on as in ordinary bouillon. After a week or longer the amount of growth in all tubes was much the same. Although it was chiefly a bottom growth in the (b) tubes, shaking produced a heavy turbidity.

To determine the effects of growth on the fate of the agglutinins, cultures in serum-bouillon were passed through Berkefeld filters. About 0.1 per cent dextrose and 10 per cent distilled sterile water were added to the filtrate to make up for losses and to effect a reduction of the pH through the action of the culture on dextrose. Such filtered fluid was inoculated with both (a) and (b) strains with the following outcome in 24 hours.

1. Serum-bouillon a-a showed no coherent disc-like deposit. The fluid was uniformly clouded with slight, easily suspended deposit.
2. Serum-bouillon a-b had only a bottom growth. This was granular and lumpy when suspended after shaking.
3. Serum-bouillon b-a had a firm, disc-like deposit. Fluid still clear.
4. Serum-bouillon b-b had a granular deposit and clear supernatant fluid.

In 48 hours (3) had become clouded. In 6 days (1) had become heavily clouded. No coherent growth in bottom. (2) was finely clouded with some surface growth. Deposit consists of lumps up to $1\frac{1}{2}$ mm. in diameter. Heavily turbid after shaking. (3) had become moderately clouded with persistent disc. (4) liquid still clear. When shaken the fluid cloudy with clumps up to 3 mm. in diameter.

A second successive filtration with the same additions as after the first filtration was carried out, the fluid tubed in 5 cc. amounts and inoculated with (a) and (b) strains. In the b-a-b tubes a deposit appeared but the supernatant fluid remained clear. When the tubes were shaken the clouding was much less pronounced than in the original cultures. Clumping in groups of 10 to 20 rods was demonstrated with the microscope. The a-b-a tubes were uniformly clouded without deposit. A microscopic examination showed no clumping.

A third filtration was carried out and the filtrate inoculated with (b). The b-a-b-b tubes showed growth in the form of a deposit. The fluid remained clear as heretofore. Even after shaking these tubes were clear on the following day.

The successive cultures in filtrates of serum-bouillon brought out the following facts: The (a) agglutinin was removed by the (a) but not by the (b) type. The (b) agglutinin was present in such amounts that three successive growths of (a) or (b) failed to remove it entirely. The (a) agglutinin forms a very cohesive mass of bacteria which is not broken up by repeated and vigorous shaking. In a normal NaOH solution the mass tends to soften and disintegrate and nearly disappear. In 5 per cent acetic acid it shrinks into a tough leathery membrane of much smaller bulk. Tested with the usual agglutinin technique, the b-a-b filtrate produced only a slight clumping of (b) in a dilution of 1/2 which was equivalent to a 1/20 dilution of the serum itself, thus indicating that the (b) agglutinin had been nearly used up. As a further control a normal cow serum was treated in the manner described, *i.e.* diluted with bouillon 1:10 and inoculated with Types (a) and (b). The (a) fluid became heavily clouded with the usual deposit. When shaken the fluid became more heavily turbid, without any clumping. A disc was not formed. The (b) fluid was at first clear with a bottom growth. Later the fluid became clouded as if the agglutinin present had been used up.

The above cultures were passed through Berkefeld filters and after adding 0.1 per cent dextrose inoculated with (a) and (b). (a) became uniformly and heavily clouded. In (b) a heavy deposit of clumps formed and the clouded supernatant fluid contained both clumps and free forms. Even in normal cow serum-bouillon the (b) agglutinins survived one multiplication of the bacilli.

The Direct and Reciprocal Absorption of Protective Antibodies.—The cow sera A and B tested for agglutinins as described above were also used in the following studies to determine their protective value. Cow A treated with living cultures and Cow B treated with heated cultures of (a) furnished sera of nearly the same protective titer. B was slightly higher and was capable of keeping alive guinea pigs receiving the surely fatal dose (usually $1\frac{1}{8}$ to $1\frac{1}{2}$ the minimum fatal dose) in amounts of 0.005 cc. to 0.01 cc.

The following experiment was carried out twice with entirely concordant results. Only the second is given.

20 cc. tubes of bouillon containing 10 per cent by volume of the immune serum of B were prepared. These were inoculated with *B. coli* (a) and (b). For controls a tube not inoculated was carried along with them and in addition a strain of *B. coli* (1085a) with capsule but without any agglutinative relation to (a). After 50 hours incubation the four tubes were refrigerated and filtered next day through separate Berkefeld filters. The filtrates were tested on guinea pigs to determine protective capacity. Each filtrate was mixed with 24 hour bouillon cultures of *B. coli* (a) and injected into the peritoneal cavity of guinea pigs. In the tests the weights of the guinea pigs were maintained between 350 and 375 gm. The bouillon for culturing was the same throughout. The culture dose was about $1\frac{1}{4}$ to $1\frac{1}{2}$ times the surely fatal dose. The outcome of the test was as follows:

The control serum-bouillon (incubated and filtered with the rest) protected in 0.5 cc., not in 0.3 cc. and 0.4 cc. doses[4]

TABLE I.

The Effect of Multiplication of B. coli (a), (b) and x in 10 Per Cent Immune Serum-Bouillon (a).

Serum used	Per cent serum in bouillon	Growth in serum-bouillon	Minimum protecting dose of serum
			cc.
Cow B	(Original undiluted serum)		0.01
"	10	Control	0.05
"	"	*B. coli* (a)	>0.30
"	"	" (b)	0.05
"	"	" x	0.07

The (a) serum-bouillon filtrate did not protect in 1.5, 2 and 3 cc. doses.[4]

The (b) serum-bouillon filtrate protected in 0.5 and 0.6 but not in 0.4 cc. doses.[4]

The control (1085a) serum-bouillon filtrate protected in 0.7 but not in 0.3 and 0.6 cc. doses. In Table I the serum present in the serum-bouillon is given in the last column.

The (a) strain of *B. coli* removed the protective substance so that 3 cc. serum-bouillon failed to lengthen the life period of the guinea pigs. It thus contained less than 1/6 of the protective substance in the control serum-bouillon. The (b) mutant removed practically none, the other strain of *B. coli* a little. This latter strain was nearly as

[4] To determine the actual serum present divide by 10.

virulent as the (a) strain. It will be noticed that the 10 per cent serum-bouillon control lost more or less during incubation and subsequent filtration. The serum in it protected in 0.05 cc. doses whereas the original serum protected in 0.01 cc. doses or less.

The Content of a (b) Antiserum in Agglutinin and Protective Antibody. —The serum of Cow C (treated with heated (b) mutant) agglutinated the mutant completely up to 1/1,280. At 1/10,240 the microscope still showed about one-third of the rods in clumps. Culture (a) was

TABLE II.

Protective Action of Immune (Cow) Serum (b).

Guinea pig No.	Dose of *B. coli*	Dose of serum	Result
	cc.	*cc.*	
1 (control)	0.06 (a)	—	Dies in 6 hrs.
2	0.07 (a)	0.5	" " 9 "
3	0.07 (a)	1.0	" " 9 "
4	1.3 (b)	—	" " 6½ "
5	1.4 (b)	0.01	" " 16 "
6	1.4 (b)	0.02	Lives

TABLE III.

The Effect of Multiplication of B. coli (a) and (b) in 10 Per Cent Immune Serum-Bouillon.

Guinea pig No.	Dose of *B. coli* (b)	Dose of 10 per cent serum-bouillon	Result Dies in	Amount of serum (calculated)
	cc.	*cc.*	*hrs.*	*cc.*
1	1.3	—	9	—
2	1.4	2 (a)	24	0.2
3	1.4	2 (b)	23	0.2
4	1.5	—	14±	—
5	1.5	3 (a)	14±	0.3
6	1.5	3 (b)	14±	0.3

slightly acted on in low dilutions only. Thus in a 1/2 or a 1/4 dilution the microscope showed chains of 4 to 8 rods among free individuals. About one-fourth of all rods were in short chains. No macroscopic agglutination was recognizable however in these or in the higher dilutions.

The protective action of the serum of Cow C was tested against

both the original *B. coli* (a) and its mutant (b). Omitting the numerous tests for determining the minimum fatal doses of both (a) and (b) and the neutralizing dose of serum we present the final tests in Table II.

It will be seen from Table II that the serum of Cow C treated with mutant (b) failed to protect guinea pigs towards (a) in a dose of 1 cc.[5] whereas 0.02 cc. protected against the surely fatal dose of (b). As has been stated, the (a) serum of Cow B protected against the surely fatal dose of both (a) and (b) in the same small amount of 0.01 cc.

To determine the absorptive capacity of (a) and (b) respectively with reference to the (b) immune serum a 10 per cent serum-bouillon was inoculated with (a) and (b), incubated for 46 hours and filtered. The filtrates were tested for residual protective substances on guinea pigs.

It will be seen from Table III that enough antibody had been removed by both (a) and (b) strains so that 0.3 cc. serum did not even retard death. Higher concentrations were not tried because of the bulk of the serum-bouillon filtrate to be injected. Unfortunately a control serum-bouillon was not carried through, but judging from the result given in Table I we may allow the surely protective dose of the serum-bouillon itself to be 5 times 0.02 cc. or 0.1 cc. serum.

DISCUSSION.

The experiments described point to the existence of two agglutinins, one directed towards the capsule and visualized in the mucoid disc-like mass formed during growth in serum-bouillon, the other directed against the naked bacteria represented by mutant (b). The (a) agglutinin is produced *in vivo* with difficulty, the (b) agglutinin readily and abundantly. The (a) antigen produces both (a) and (b) agglutinins, the (b) antigen only (b) agglutinins. The mutant (b) fails to remove (a) agglutinins while multiplying in (a) serum-bouillon whereas (a) does. These actions are visualized in the agglutinin disc which is absent in culture filtrates of serum-bouillon following the growth of (a) but is still present after the growth of (b).

The protective antibodies, as tested on guinea pigs by mixing serum and living culture and injecting into the peritoneal cavity, follow closely the agglutinins. The (a) serum protects against both (a) and

[5] Higher doses not tried.

(b) forms of the homologous *B. coli* strain, the (b) serum only against (b). Both (a) and (b) strains remove or bind about the same amount of protective antibody in the (b) serum of Cow C. The abundance of (b) agglutinins produced by (a) in Cows A and B is shown by the fact that three repeated crops of *B. coli* in serum-bouillon failed to exhaust the fluid of all (b) agglutinin. A similar abundance of (b) agglutinin was produced in Cow C treated only with mutant (b).

In the preparation of antisera both living and heated (62°C.) cultures of the (a) form were of equal value when injected into cows. The few animals used do not permit generalizations, but it appears that the heated cultures were less injurious to the animal and produced antibodies more promptly perhaps because of the much larger numbers of bacilli that could be injected. Attention is called to the fact that *B. coli* is able to multiply abundantly in the first and second serum-bouillon filtrates of its own growth, less so in the third. After each filtration about 0.1 per cent dextrose was added to lower the pH of the culture fluid through the formation of acid by *B. coli*.

The formation of a coherent viscid disc-like mass as a result of agglutination appears to be associated with capsulated bacteria.

Wadsworth and Kirkbride[6] in testing a pneumococcus serum of Type III observed a prompt agglutination in a 1/1 concentration. "Within 15 to 30 minutes . . . . a loose cap had formed similar to those often seen in tests with Type I and Type II." Clumping was seen no higher than in 1/10 dilutions. Coulter[7] in preparing immune rabbit sera against Friedländer strains observed instant coarse flocculation settling down to a compacted disc in bottom of tube in serum dilutions of 1/1 and 1/5. Small and Julianelle[8] observed the same phenomenon in the agglutination of various strains of *B. mucosus capsulatus*. Avery and Morgan[9] describe antipneumococcus sera when acting on the specific soluble substance of Type II as producing in low dilutions a compact disc-like precipitate, in higher dilutions only flocculation.

The difficulty of producing or failure to produce antibodies towards capsulated strains has been observed and commented on by many working with such forms for the past 30 years. It would seem that

[6] Wadsworth, A. B., and Kirkbride, M. B., *J. Exp. Med.*, 1917, xxv, 629.
[7] Coulter, C. B., *J. Exp. Med.*, 1917, xxvi, 763.
[8] Small, J. C., and Julianelle, L. A., *J. Infect. Dis.*, 1923, xxxii, 456.
[9] Avery, O. T., and Morgan, H. J., *J. Exp. Med.*, 1925, xlii, 347.

such antibodies can be produced by using large, resistant animals and continuing the treatment over long periods. It may seem advisable to compare the horse and the cow with respect to this problem.

It has been noted in a previous paper[10] that the immune serum prepared with the *B. coli* strain (a) failed to protect guinea pigs against certain bovine types of *B. coli* and did protect against others but in doses about 50 times larger than that required against the homologous strain. Recently a highly viscid strain of *B. coli* was isolated from the spleen of a presumably normal guinea pig. The strain failed to ferment saccharose, being in this respect like (a). The viscidity was extreme. Cobweb-like threads several feet long were brought out of the culture tube with the loop and had to be burned off. Early mutation did not take place on agar plates. The intraperitoneal minimum fatal dose of a 24 hour bouillon culture was 0.2 cc. 0.25 cc. culture plus 0.5 cc. serum of Cow B (heated (a) culture treatment) protected. In serum dilutions of 1/2 to 1/20 of Cow B the bacilli were clumped into a floating, gelatinous mass. A certain limited relationship between the capsular material of this form and that of the bovine form is thus demonstrated.

The value of relationships based solely on serologic determinations is somewhat impugned by the above results. If (a) and (b) had been isolated independently, (b) would have been regarded as different from (a) since (b) serum fails to agglutinate (a), to absorb (a) agglutinins and to protect animals against (a). On the other hand (a) serum protects against (b) and agglutinates (b) in high dilutions. Such serologic relationships of bacteria morphologically and culturally alike but obtained from different sources may be regarded provisionally as indicating mutation or degradation of one type or strain (mutant) from the other (original). The taxonomic value of agglutinins obviously must depend on what is agglutinated. The capsular material, being probably developed by parasitism, is the last character to be acquired and most easily lost, as is evidenced by the rapid mutation on agar plates, during which process the capsule disappears. Since the fermentation reactions of (a) and (b) are not changed, it seems reasonable to regard them as of more fundamental value in

[10] Smith, T., *J. Exp. Med.*, 1927, xlvi, 141.

this group in classification than what is denominated the type-specific substance, which seems to be related to or identical with the capsular substance.

It has been stated in a previous paper[11] that immune cow serum does not exercise any appreciable influence on the toxic effect of culture filtrates of (a) and (b) when mixed with them and injected into the jugular vein of calves. It would have been of interest to determine quantitatively if any toxin-neutralizing action could be attributed to the sera of highly immunized cows. Owing to the difficulty of obtaining calves in sufficient numbers and of the same breed at any one time, no accurate comparative quantitative toxin-neutralizing experiments have been made.

CONCLUSION.

The relation between a strain of *B. coli* and its mutant with reference to the production of agglutinins and protective antibodies may be expressed by the statement that the original strain when injected into cows develops antibodies both towards itself and the mutant whereas the mutant produces them only towards itself. The results point to the capsular substance as the material carrying virulence or, expressed somewhat differently, the factor which protects the microorganism in the host.

[11] Smith, T., and Little R. B., *J. Exp. Med.*, 1927, xlvi, 123.