THE SIGNIFICANCE OF AGGLUTININS IN THE IMMUNITY OF THE RABBIT TO THE HOG-CHOLERA BACILLUS.

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(Received for publication, May 18, 1917.)

A great deal of experimental work has been done on agglutinins, yet we have very little evidence as to the part they play in immunity to any given infectious disease. Much of the work tends to show that they are not indicators of immunity but in spite of this the agglutinin titer is often used as an index of immunity after antityphoid vaccination and when the titer falls it is regarded as an indication for revaccination. In the production of various immune sera it is often assumed that the height of the agglutination titer is an index of the antibody content but the evidence on which this assumption is based is not clear. We have endeavored to throw some light on the relation of agglutinins to immunity by the use of the highly virulent culture of the hog-cholera bacillus described in the previous paper (1).

A large part of the experimental work on agglutinins has been done with the rabbit and the horse inoculated with the typhoid bacillus, and the great weakness of this work is that in the former animal at least, this organism does not produce a true invasive disease but acts only when large numbers of bacteria are given. On the other hand, the hog-cholera bacillus produces, in the rabbit, a true disease resembling in many respects typhoid fever in man.

HISTORICAL.

In 1894 Smith and Moore (2) showed that heated cultures of the hog-cholera bacillus would not produce an immunity in the rabbit but that the injection of a living attenuated culture would produce an active immunity to the more virulent organism. They did not at this time study the agglutinins, but later Smith and Reagh (3) produced agglutinins by the injection of heated as well as living cultures of the hog-cholera bacillus.

Shoukévitch (4) reports work similar to that to follow in which, using the hog-cholera bacillus and the rabbit, he showed that the injection of heated cultures caused an increase in the complement-fixing bodies, agglutinins, and opsonins but that this was not associated with an immunity. The injection of a living culture of low virulence caused a very slight production of these bodies but did cause an active immunity to more virulent organisms. Of fifteen animals tested in this way, five showed no effects, one died with a typical septicemia, two died from acute intoxication, and seven died in from 15 to 70 days with a paralysis of various groups of muscles. All showed little or no increase in the immune bodies following the injection of the culture of low virulence.

Whether the phenomenon described by Bull (5) under the name of *intra vitam* agglutination is related to *in vitro* agglutination is a question that will not be considered in this paper but we will consider the relation of this phenomenon to immunity to the hog-cholera bacillus. Bull has recorded experiments with a rather large series of organisms, the avirulent members of which are clumped when injected intravenously into normal rabbits, while the virulent members are not clumped and are found in the circulation for some time after the injection. When the bacilli are clumped they rapidly disappear from the blood stream and in a half hour after the injection, films from the liver and lungs show many polymorphonuclear leukocytes filled with organisms. Virulent organisms are not clumped in the circulation of normal rabbits but are rapidly clumped and phagocyted when injected into immune animals. Bull says (5) "The degree of agglutination and opsonization of bacteria within the animal body is inversely parallel to the infectiousness of the bacteria for the host" but he is careful to state that exceptions may be found to this rule.

EXPERIMENTAL.

Cultures Used.—Reference has already been made to the highly virulent test culture (1). The parent culture that had not been passed through rabbits and having the laboratory number XII was used as a vaccine and in small doses of the living culture to produce an immunity.

Hog-cholera Neb., isolated by Dr. Smith in 1886 (6), is now a culture of very low virulence. It is a typical hog-cholera bacillus except for its lessened virulence.

Hog-cholera Ark., isolated by Dr. Dinwiddie in 1889, was referred to by Dr. Smith in 1903. (3) and the cultural characters have been reported more recently (7). It is an organism of moderate virulence, probably due in part at least to its prolonged cultivation on artificial media.

Use of Heated Cultures.

The first experiment was made with a vaccine prepared by suspending the 24 hour growth from an agar slant inoculated with Hog-cholera XII in 10 cc. of salt solution and heating for 1 hour at 60°C. The heated suspension was incubated over night and several loops were transferred to bouillon in order to be sure that it was sterile. Having proven that all the bacteria had been killed the vaccine was injected subcutaneously into two rabbits of approximately equal weight. The first injection was of 0.5 cc. and was followed in 5 days by 1 cc. 6 days later they were each given 2 cc. Both rabbits bore the injections well, showing only a slight and temporary loss in weight.

18 days after the beginning of the treatment and 7 days after the last inoculation these two rabbits, together with a control, were bled from the ear vein and the serum of the inoculated animals was tested for agglutinins against the strain of the hog-cholera bacillus with which they had been injected and also against the strain that had been passed serially through rabbits and with which they were to be inoculated in order to test their immunity.

In order that the rabbits might have every opportunity to show any immunity they might have gained by the injection of the heated cultures they were tested by the cutaneous method, two drops of a 24 hour bouillon culture being lightly rubbed into the shaven skin of each animal. This test was made 2 days after the bleeding from the ear vein and 9 days after the last injection of the heated cultures. The results of this experiment are summarized in Table I.

TABLE I.

Test of the Power of Heated Cultures to Produce Agglutinins and Immunity.

Rabbit No.	Vaccinated.	Limit of agglutination* for Hog-cholera XII.		Weight.	Immunity test. Result of cutaneous inoculation with test culture.
		Stock.	Passage strain.		
				gm.	
1	Three times with heated culture.	$\frac{1}{12,800}$	$\frac{1}{12,800}$	1,823	Died in 5 days.
2	Same as No. 1.	$\frac{1}{12,800}$	$\frac{1}{12,800}$	1,889	" " 7 "
3	Control.	Not tested.		2,006	" " 6 "

* The limit of agglutination is the highest dilution in which clumps of bacteria can be seen with the naked eye. A 24 hour bouillon culture was used as an antigen throughout.

It will be seen that while both treated rabbits had in their serum agglutinins that could be demonstrated in a dilution of $\frac{1}{12,800}$ they

had no immunity. Both died in approximately the same number of days after the inoculation as did the control. At autopsy all three rabbits showed the same local as well as visceral lesions, the only essential difference being that both vaccinated rabbits had a small area of pneumonia. It is quite possible, however, that the latter was due to another organism that was affecting our stock rabbits.

A similar experiment, but with results that are not so clear-cut, was made some time later. The preparation of the vaccine and the dosage was the same as in the preceding experiment except that the culture used was the highly virulent rabbit passage strain. The test for immunity was made by injecting subcutaneously 0.0000001 cc. of a 24 hour bouillon culture of the passage strain. The results are summarized in Table II and show that of the vaccinated rabbits the one that had the higher agglutination titer died, while the other one survived without showing any marked effects from the inoculation. Examination of the controls shows that we were using about the smallest dose that would cause an infection, so that the apparent immunity of the one vaccinated rabbit might be due to causes other than the injected vaccine such as a natural immunity or too small an infecting dose. In spite of this discordant result it is clear that there was no demonstrable immunity in the rabbit with the higher agglutination titer.

TABLE II.

Test of the Power of Heated Cultures to Produce Agglutinins and Immunity.

Rabbit No.	Vaccinated.	Limit of agglutination for test culture.	Weight.	Subcutaneous injection of test culture.	
				Amount.	Result.
			gm.	*cc.*	
4	Three-times with heated culture.	$\frac{1}{25,600}$	2,011	0.0000001	Died in 9 days.
5	Same as No. 4.	$\frac{1}{12,800}$	2,030	0.0000001	No effect.
6	Control.	0	2,683	0.0000001	Died in 9 days.
7	"	0	1,763	0.00000001	No effect.

Use of Living Cultures to Produce Immunity.

The immunization of rabbits by the injection of living cultures of the hog-cholera bacillus is very difficult, for their resistance may be overcome by the injection of too large doses or they may succumb to

some spontaneous disease to which they seem very susceptible during such treatment. We did succeed in getting four animals to the stage where they could be tested against the highly virulent culture and as the treatment of each animal is different from the others a summary of the immunization is given below.

Rabbit 8.—Three injections of a non-virulent strain followed by a mildly virulent strain.

June 6, 1916. Subcutaneous injection of 0.01 cc. of a 24 hour bouillon culture of Hog-cholera Neb. Slight rise in temperature but no loss in weight.

June 20. Subcutaneous injection of 1 cc. of a 24 hour bouillon culture of Hog-cholera Neb. Loss in weight but no increase in temperature.

July 8. Intravenous injection of 0.1 cc. of a 24 hour bouillon culture of Hog-cholera Neb. Rise in temperature but no loss in weight. Another rabbit that had had the same injections died during the night following the intravenous inoculation.

July 21. Subcutaneous injection of 0.1 cc. of a 24 hour bouillon culture of Hog-cholera Ark. No loss in weight and no rise in temperature. Previous tests had shown that this culture in this amount would kill a normal animal in 20 days.

This rabbit was bled three times after the last inoculation and the results of the agglutination tests are given in Table III.

TABLE III.

Agglutination Titer of the Serum of Rabbit 8.

Culture agglutinated.	Limit of agglutination for serum drawn on.		
	Aug. 4	Sept. 19	Oct. 3
Hog-cholera XII	1/80,000	1/51,200	1/3,200
" XII, passage series	1/20,000	1/25,600	1/6,400

Rabbit 9.—Injected with increasing numbers of Hog-cholera Ark. (mildly virulent strain).

June 6, 1916. Subcutaneous injection of 0.0001 cc. of a 24 hour bouillon culture. Rise in temperature but no loss in weight.

June 20. Subcutaneous injection of 0.01 cc. of a 24 hour bouillon culture Rise in temperature and loss in weight extending over a period of 20 days.

July 21. Subcutaneous injection of 0.1 cc. of a 24 hour bouillon culture. Slight loss in weight but no rise in temperature.

This last injection was one that would kill the normal rabbit, so no further injections were given but the animal was allowed to rest until it was tested for immunity to the highly virulent strain. During this period of rest it was bled three times and the limit of agglutination for the hog-cholera bacillus determined. The results of these tests are given in Table IV.

TABLE IV.

Agglutination Titer of the Serum of Rabbit 9.

Culture agglutinated.	Limit of agglutination for serum drawn on.		
	Aug. 4	Sept. 19	Oct. 3
Hog-cholera XII	1/20,000	1/3,200	1/1,600
" XII, passage series	1/5,000	1/1,600	1/1,600

Rabbit 10.—Injected with increasing amounts of Hog-cholera XII (virulent culture).

June 20, 1916. Subcutaneous injection of 0.0000001 cc. of a 24 hour bouillon culture. Rise in temperature but no loss in weight.

July 11. Injection of June 20 repeated. No rise in temperature and no loss of weight.

July 21. Subcutaneous injection of 0.00001 cc. of a 24 hour bouillon culture. No loss in weight or rise in temperature.

Aug. 7. Subcutaneous injection of 0.001 cc. of a 24 hour bouillon culture. No effect on weight or temperature. Control rabbit died in 4 days.

Aug. 19. Subcutaneous injection of 0.01 cc. of a 24 hour bouillon culture. No effect on weight or temperature. The animal was now allowed to rest for some time before its resistance to the highly virulent strain was tested. Table V gives the results of the agglutination tests made during this period.

TABLE V.

Agglutination Titer of the Serum of Rabbit 10.

Culture agglutinated.	Limit of agglutination for serum drawn on.		
	Aug. 31	Sept. 17	Oct. 3
Hog-cholera XII	1/25,600	1/25,600	1/6,400
" XII, passage series	1/25,600	1/25,600	1/6,400

Rabbit 11.—One injection of a sublethal number of Hog-cholera XII bacilli (virulent culture).

Aug. 22, 1916. Subcutaneous injection of 0.00001 cc. of a 24 hour bouillon culture of Hog-cholera XII. Marked loss in weight and rise in temperature. Another rabbit inoculated with the same amount of culture at the same time died in 12 days. In Table VI are given the results of the agglutination tests made on the serum of this rabbit previous to its inoculation with the highly virulent culture.

TABLE VI.

Agglutination Titer of the Serum of Rabbit 11.

Culture agglutinated.	Limit of agglutination for serum drawn on.	
	Sept. 18	Oct. 3
Hog-cholera XII	1/6,400	1/3,200
" XII, passage series	1/3,200	1/3,200

On Oct. 3 these four rabbits together with two controls were bled from the ear vein and the sera tested for agglutinins to the highly virulent culture. 3 days later each was given a subcutaneous injection of 0.000001 cc. of a 24 hour bouillon culture of the highly virulent organism in order to test their immunity. The necessary data for the understanding of this test together with the results are given in Table VII. The subcutaneous route was chosen for the inoculation in order to be sure that all the animals received the same amount of culture.

Examination of the table will show that the rabbits previously injected with living cultures were not affected when inoculated with the highly virulent culture, whereas the controls died in 7 days. The agglutination titer of the sera of each of these immune animals was below that found in the serum of the animals injected with heated cultures, yet the latter promptly succumbed to an inoculation with this virulent culture in small amounts.

3 months later two of these immune rabbits were again tested for agglutinins and then injected with a large amount of the highly virulent culture. The results are summarized in Table VIII where it will be seen that one rabbit did not survive this severe test while the animal with the lower titer resisted one thousand times the minimal fatal dose.

TABLE VII.

Test of the Power of Living Cultures to Produce Agglutinins and Immunity.

Rabbit No.	Immunization.				Test of immunity to Hog-cholera XII, passage series, on Oct. 6, 1916.			
	Date of injection.	Strain of hog-cholera.	Amount of 24 hr. bouillon culture injected.	Route.	Time after last injection.	Weight.	Agglutination titer 3 days before test of immunity.	Result of subcutaneous injection of 0.000001 cc. of 24 hr. bouillon culture.
	1916		*cc.*		*days*	*gm.*		
8	June 6	Neb.	0.01	Subcutaneous.	77	3,028	1/6,400	No effect.
	" 20	"	1.0	"				
	July 8	"	0.1	Intravenous.				
	" 21	Ark.	0.1	Subcutaneous.				
9	June 6	Ark.	0.0001	Subcutaneous.	77	3,374	1/1,600	No effect.
	" 20	"	0.01	"				
	July 21	"	0.1	"				
10	June 20	XII	0.0000001	Subcutaneous.	48	2,768	1/6,400	No effect.
	July 11	XII	0.0000001	"				
	" 21	XII	0.00001	"				
	Aug. 7	XII	0.001	"				
	" 19	XII	0.01	"				
11	Aug. 22	XII	0.00001	Subcutaneous.	45	2,024	1/3,200	No effect.
12	Normal animal for control.				0	2,932	0	Died in 7 days. Typical lesions.
13	Normal animal for control; injection one-tenth the amount given other rabbits.				0	2,359	0	Died in 7 days. Typical lesions

The results of these tests clearly indicate that an animal may show agglutinins to the hog-cholera bacillus *in vitro* and yet have no immunity. It cannot be said, however, that these bodies have no relation to immunity for they are present in the sera of all the immune animals though, at the time of the test, not in as high dilutions as in the vaccinated rabbits.

TABLE VIII.

Test of the Power of Living Cultures to Produce Agglutinins and Immunity.

Rabbit No.	Immunization.	Agglutination titer 3 days before test of immunity.	Test of immunity.	
			Amount of 24 hr. bouillon culture injected.	Result.
			cc.	
8	See Table VII.	$\frac{1}{1,280}$	0.0001	Died in 8 days.
11	" " VII.	$\frac{1}{640}$	0.0001	Slight rise in temperature.
6	Control.	0	0.0000001	Died in 9 days.
7	"	0	0.00000001	No effects.

Attempts have been made to differentiate after the method of Joos (8) the agglutinins in the vaccinated rabbit from those found in the immune animals. No difference has been found in the susceptibility to heat of the agglutinins from these two sets of animals nor do the sera act differently on heated bacteria.

Intra Vitam Agglutination.

The work of Bull (5) suggested the comparison of the *intra vitam* agglutination in vaccinated rabbits and in those immunized by the use of living cultures. When a suspension of the living organisms from the highly virulent strain are injected intravenously into either of these animals they are promptly clumped and rapidly disappear from the circulation. Films made from the liver half an hour after the injection show cells packed with bacteria. Most of the phagocytic cells found were polymorphonuclear leukocytes but a few phagocytic endothelial cells were also present. No difference was noted in the reactions of animals from the two sets though one was immune and the other was very susceptible to the bacterium injected. The most interesting phase of this work was that the control animals also showed typical *intra vitam* clumping. This fact was verified repeatedly but only one experiment will be given.

The growth from two 24 hour agar slants inoculated with Hog-cholera XII passage virus was suspended in 15 cc. of salt solution. Shaken and centrifugal-

ized for ½ hour. Removed the supernatant fluid and suspended the residue in 5 cc. of salt solution and shook vigorously to break up clumps. Injected intravenously into a normal rabbit weighing 2,270 gm. Blood removed from the heart at stated intervals and dilutions made for plate cultures. The first dilution was made in a glass-stoppered bottle and was shaken for some time to break up clumps. At the same time films were made that later were stained with Manson's methylene blue. The findings in the films as well as the results of the plate counts are given in Table IX.

40 minutes after the injection, the rabbit was chloroformed and films were made from the liver. Cells containing bacteria were present but were not numerous.

TABLE IX.

Intra Vitam Agglutination Test Using a Normal Rabbit.

Time after injection.		Bacteria per cc. in heart's blood.	Result of examination of film.
min.	*sec.*		
0	31	30,000,000	Large clumps of bacteria embedded in a blue-staining homogeneous mass.
2	30	4,800,000	No bacteria found.
5	24	5,950,000	" " "
15	0	75,000	" " "
30	0	117,000	" " "

This experiment, as well as the others made, shows that in the normal rabbit there is a prompt clumping of the injected bacteria, a rapid disappearance from the blood stream as shown by films and plate counts, and a phagocytosis of the bacteria by cells in the liver and in other organs. The centrifugalization of the bacteria had nothing to do with the clumping for it occurred in other animals where a suspension made directly from the agar slant was injected. When the dilutions of blood were not shaken in glass-stoppered bottles, plates made 30 minutes after the injection and containing $\frac{1}{500}$ cc. of blood were sterile.

SUMMARY AND CONCLUSION.

Rabbits may show a high agglutination titer to the hog-cholera bacillus and have no immunity and on the other hand immune animals may have a comparatively low agglutination titer. In other words, with this organism the height of the agglutination titer does

not indicate the degree of immunity. As this bacillus so closely resembles the typhoid bacillus biologically and pathologically, it seems safe to conclude, until evidence is brought forth to the contrary, that in man the height of the agglutination titer does not indicate the actual degree of immunity to the latter organism. The same would apply to other members of the typhoid-colon group. It would not be wise to draw a more general conclusion until other organisms have been tested. This does not mean that agglutinins are not related to immunity but it brings up the question of the wisdom of using them as a guide in immunization with the colon-typhoid group.

When injected into the normal, vaccinated, or immune rabbit, the virulent hog-cholera bacillus is rapidly clumped and disappears from the circulation. 40 minutes after injection these organisms can be found in phagocytes in the liver. The fact that the normal rabbit gives this *intra vitam* agglutination is an exception to the findings of Bull that virulent organisms remain in the circulation for some time after injection.

BIBLIOGRAPHY.

1. TenBroeck, C., *J. Exp. Med.*, 1917, xxvi, 437.
2. Smith, T., and Moore, V. A., *U. S. Dept. Agric., Bureau Animal Industry, Bull. 6*, 1894, 41.
3. Smith, T., and Reagh, A. L., *J. Med. Research*, 1903, ix, 240.
4. Shoukévitch, J., *Ann. Inst. Pasteur*, 1910, xxiv, 728.
5. Bull, C. G., *J. Exp. Med.*, 1916, xxiv, 25.
6. Smith, T., *U. S. Dept. Agric., Ann. Rep., Bureau Animal Industry*, 1886, 38.
7. TenBroeck, *J. Exp. Med.*, 1916, xxiv, 213.
8. Joos, A., *Centr. Bakteriol., 1te Abt., Orig.*, 1903, xxxiii, 762.