THE REMOVAL OF AGGLUTININ FROM SENSITIZED MOTILE BACTERIA

SECOND PAPER. THE AGGLUTINATIVE PROPERTIES OF WATER WASHINGS

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Observations on the removal of agglutinin from sensitized motile bacteria were presented and discussed in a preceding paper.[1] Extraction of the sensitized test organism (*Bacillus aertrycke*) in a 5 per cent sodium chloride solution at a temperature of 60°C. resulted in the removal of considerable flagellar agglutinin while the somatic agglutinin was practically unaffected. Brief mention was made of an anomalous reaction which occurred when distilled water was employed as the extracting or washing medium. The reaction is described in detail in the following experiments together with some additional observations leading to a possible explanation.

General Methods

A normal actively motile strain of *B. aertrycke* (guinea pig paratyphi, Type II) recently isolated from the spleen of a naturally infected guinea pig was used as the test organism. Cultivation was carried out on moist Blake bottles, inoculated with a sufficient amount of young broth culture to cover the surface of the agar and incubated at 37°C. for 18 hours. Cultivation on a dry medium lowers the floccular agglutinability of the bacteria. The growth was removed with normal saline, transferred to a graduated centrifuge tube, and sedimented for an hour. The supernatant was discarded and the bacteria, still in a packed state, were rinsed once with saline.

The serum was from a rabbit immunized with the above strain of *B. aertrycke* and it contained both flagellar and somatic agglutinins. For sensitization of the bacteria the following materials were used: 0.5 cc. of packed bacteria, 0.1 cc. of

[1] Nelson, John B., *J. Exp. Med.*, 1928, **48**, 825.

antiserum, 2.4 cc. of saline. This formula gives the minimum proportion of packed bacteria required for the nearly complete removal of flagellar agglutinin with the serum dilution employed. It was about twice the amount required in the preceding work[1] with a different strain of *B. aertrycke* and its homologous antiserum of approximately the same titer. The bacteria were resuspended and the mixture incubated at 37°C. for 5 hours followed by overnight refrigeration.

The mixture was now centrifuged for 1 hour at the same speed and the supernatant removed. The packed sensitized bacteria were suspended in 2.5 cc. of distilled water and again centrifuged for an hour. The washing process was continued 3 to 5 times with fresh water after each sedimentation.

The agglutinin content of the absorbed antiserum and of the washings was determined by macroscopic agglutination with 0.5 cc. of antigen and 0.5 cc. of fluid dilutions. As a control, unabsorbed antiserum diluted and incubated similarly to the absorbed serum was tested in the same way. Two antigens designated whole and heated were used. The former was a fresh saline suspension of *B. aertrycke* and contained both flagellar and somatic components. The latter was a saline suspension heated to 100°C. for 30 minutes, washed, and resuspended in saline. It contained only the somatic component. Both suspensions were standardized to 2.4 with the Gates apparatus. Incubation was carried out at 37°C. for 3 hours followed by overnight refrigeration.

Experiments

The results of a typical experiment with the use of distilled water as the medium for the removal of agglutinin are given in Table I. The agglutinin titer of the absorbed antiserum mixture and of the several water washings is compared with that of the unabsorbed antiserum. If we take into account the initial dilution of the unabsorbed and absorbed antiserums, the actual titers are 25 times the figure given.

The unabsorbed antiserum agglutinated the whole antigen in high dilution. The type of clump was mixed, though predominantly flocular in the lower dilutions, while in the higher dilutions it was purely floccular. It agglutinated the heated or deflagellated antigen in the lower dilutions only with a purely granular type of clump. While the limit of agglutination for the whole antigen was identical with that of the antiserum used in the preceding work,[1] the intensity of the reaction in the lower dilutions was less with the present antiserum. The limit of agglutination for the heated antigen was one dilution higher with the earlier antiserum, and the intensity of the reaction was likewise more marked. The absorbed antiserum gave a slight, floccular agglu-

tination in very low dilution with whole antigen but no reaction with heated antigenen the lowest dilution possible. The single absorption afforded an approximately 99 per cent removal of both flagellar and somatic agglutinins.

TABLE I

Agglutination of B. aertrycke by Unabsorbed Antiserum, Absorbed Antiserum and the Water Washings of a Sensitized Suspension

Test fluid	Antigen	Test fluid dilutions										
		2	4	8	16	32	64	128	256	512	1,024	2,048
Unabsorbed serum	W*	++++ M	++++	++++	++++	++++	+++	+++	++	++	+	±
	H	++++ G	+++	++	+	±	±	−	−	−	−	−
Absorbed serum	W	+ F	±	trace	−	−	−	−	−			
	H	−	−	−	−	−	−	−	−			
1st water washing	W	± F	trace	−	−	−	−	−	−			
	H	−	−	−	−	−	−	−	−			
2nd water washing	W	++ F	++	+	+	+	±	±	−			
	H	++ F	+	±	±	±	−	−	−			
3rd water washing	W	++ F	++	+	+	±	±	−	−			
	H	++ F	+	±	±	−	−	−	−			
4th water washing	W	++ F	+	+	±	±	−	−	−			
	H	+ F	±	±	±	−	−	−	−			

* W = whole bacteria, H = heated bacteria (100°C.), M = mixed agglutination, F = floccular agglutination, G = granular agglutination. The same abbreviations are used in all the subsequent tables.

With the first water washing there was a slight floccular agglutination in the presence of whole antigen only. The following washings reacted quite differently. With the second washing there was a marked increase in both the intensity and the titer limit of agglutination with the whole antigen. The type of clumping was floccular. In addition there was a reduced but perfectly definite agglutination

in the presence of heated antigen. The type of clump was likewise floccular, with this difference that the aggregates were more loosely formed and became less compact upon standing than the usual floccular clumps. The subsequent washings duplicated the reaction though on an increasingly lower level.

When the above reaction is viewed as it stands, it appears that the suspension of sensitized motile bacteria in a salt-free medium results in the removal of agglutinin which gives a floccular type of agglutination not only in the presence of bacteria with their flagella attached but also in the presence of deflagellated bacteria. The occurrence of a floccular agglutination in the presence of deflagellated bacteria is contrary to the many observations which have been made on the agglutinative behavior of motile organisms.

Repetition of the above experiment with other strains of the same bacterial species and with another species of Salmonella (*B. paratyphi*, Type I), likewise of guinea pig origin, gave essentially similar results. The agglutinin titer of the second water washing fluctuated between 1:64 and 1:256 with a corresponding fluctuation in the titer of the other washings. In some cases the second and third washings were of equal titer. It may be noted that the intensity of the agglutination reaction with the water washings was regularly less marked than that of the extraction fluids in the preceding work.[1]

Upon consideration of the reaction it seemed possible that the flocculation which occurred in the presence of heated or deflagellated antigen might be due to the precipitation of material present in the washings. These, with the exception of the first, regularly showed a distinct turbidity which swirled visibly upon agitation. It was not due to bacteria, although the washings was never entirely free of bacteria. There was no sedimentation upon standing. The degree of turbidity tended to decrease with successive washings but a gradation was not always apparent. The stability of the washings in the presence of salt was determined by serial dilution with saline.

Sensitized bacteria were washed four times with distilled water and a portion of each washing heated to 70°C. for 30 minutes. The unheated and heated washings, in a total volume of 1 cc., were diluted serially with saline and incubated without the addition of antigen. The findings with the antigen-free washings are given in Table II.

There was no reaction with the first unheated washing. The others showed a graded flocculation with the settling out of a loose fluffy sediment which was easily dispersed upon agitation. There was never any flocculation with the heated washings. In appearance and amount the sediment was identical with that of the washings in the presence of heated antigen and in appearance with that of a pure flagellar suspension in the presence of specific antiserum. The material present in the washings likewise resembled flagella in its thermolability. Centrifuged suspensions of flagella, however, unlike the water wash-

TABLE II

The Reaction of Unheated and Heated Water Washings of a Sensitized Suspension upon Dilution with Normal Saline

Test fluid	State	Test fluid dilutions					
		2	4	8	16	32	64
1st water washing	Unheated	−	−	−	−	−	−
	Heated	−	−	−	−	−	−
2nd water washing	Unheated	++ F	+	±	±	±	−
	Heated	−	−	−	−	−	−
3rd water washing	Unheated	++ F	+	±	±	±	−
	Heated	−	−	−	−	−	−
4th water washing	Unheated	+ F	±	±	±	−	−
	Heated	−	−	−	−	−	−

ings, are nearly water clear. The presence of salt-precipitable material in all the washings save the first was definitely indicated.

The water washings always gave a more marked agglutination, both as to titer and to intensity, in the presence of whole antigen than in the presence of heated antigen. The reaction with the whole antigen was regarded as essentially a true agglutination due to flagellar agglutinin removed from the sensitized bacteria by the washing process. Since flagellar agglutinin is heat-stable at 70°C., at which temperature the washings no longer flocculate with saline alone, it was possible to test this assumption by heat inactivation.

Two series of 3 water washings from sensitized bacteria were employed. One series was heated at 70°C. for 30 minutes. The other was used unheated. Both were tested as usual against whole and heated antigen. The reactions with the heated washings are given in Table III. The unheated washings behaved essentially as before (Table I) and the results are not included.

The heated washings agglutinated the whole bacteria with the usual floccular clumping which was more compact than that of the typical clumping with pure flagella. As to titer, there was little difference

TABLE III

The Agglutination of B. aertrycke by the Heated Water Washings of a Sensitized Suspension

Test fluid	Antigen	Test fluid dilutions							
		4	8	16	32	64	128	256	512
1st water washing	W	++ F	±	−	−	−	−	−	−
	H	−	−	−	−	−	−	−	−
2nd water washing	W	++ F	++	++	+	+	trace	−	−
	H	−	−	−	−	−	−	−	−
3rd water washing	W	++ F	++	++	+	+	trace	−	−
	H	−	−	−	−	−	−	−	−

between the unheated and heated washings. Unlike the unheated washings, however, the heated ones failed to react in the presence of deflagellated antigen. It seems evident that the floccular reaction with whole antigen was a true agglutination due to the presence of flagellar agglutinin in the washings. That the water washings from the sensitized bacteria, with the exception of the first, contained flagella in suspension was a suggestive fact. The actual presence of flagella, under the conditions of the experiment, was difficult to demonstrate. Some observations, however, on the behavior of unsensitized motile bacteria are presented as contributing evidence.

0.5 cc. amounts of *B. aertrycke*, in centrifuge tubes, were resuspended with 2.5 cc. of distilled water and sedimented. Because of the difficulty in getting clear supernatants with unsensitized bacteria the time was increased to 90 minutes and

the speed from approximately 2,000 to 2,500 R.P.M. The bacteria were washed four times in this way. While the washings were not quite water-clear they failed to show the characteristic turbidity of the washings from sensitized bacteria. One lot was diluted serially with saline in a total volume of 1.0 cc. and incubated without antigen for the usual length of time. The other lot was diluted serially

TABLE IV

The Reaction of Unheated Water Washings of Unsensitized Bacteria upon the Addition of Antiserum and of Normal Saline

Test fluid	Dilution fluid	Test fluid dilutions						
		2	4	8	16	32	64	128
1st water washing	Immune serum	++ F	+	+	±	±	±	−
	Saline	−	−	−	−	−	−	−
2nd water washing	Immune serum	++ F	+	±	±	±	−	−
	Saline	−	−	−	−	−	−	−
3rd water washing	Immune serum	+ F	+	±	±	−	−	−
	Saline	−	−	−	−	−	−	−
4th water washing	Immune serum	+ F	±	±	−	−	−	−
	Saline	−	−	−	−	−	−	−

TABLE V

The Agglutinability of Whole and Washed Suspensions of B. aertrycke

Antigen	Serum dilutions									
	4	8	16	32	64	128	256	512	1,024	2,048
Whole bacteria	++++ M	++++	++++	+++	+++	++	++	++	+	−
Washed bacteria	++++ M	++++	+++	++	+	±	trace	trace	−	−

with saline in a volume of 0.5 cc., and 0.5 cc. of a 1:400 dilution of homologous antiserum added to each lot. Incubation was carried out in the same way. The washed unsensitized bacteria were standardized in saline and their agglutinability compared with that of unwashed whole bacteria. The same antiserum, used throughout, was employed. The observations on unsensitized bacteria are presented in Tables IV and V.

The washings from the unsensitized bacteria failed to flocculate upon the addition of saline. When dilute antiserum was added, however, flocculation occurred and the type of clumping was identical with that of the water washings from sensitized bacteria in the presence of saline. It was a typical flagellar agglutination. Unlike the series from sensitized bacteria, however, the initial washing contained flocculable material. That the bacteria present in these washings were too few in number to influence the reaction was shown by the following experiment.

Sedimented whole bacteria were rinsed and resuspended in saline. The suspension contained only bacteria; there were no free flagella. The suspension was then diluted with saline to a barely visible turbidity. A 0.5 cc. portion of this suspension was carried through five serial dilutions and 0.5 cc. of a 1:400 dilution of antiserum added to each tube. There was no agglutination in any dilution after the usual incubation.

The agglutinability of the washed unsensitized bacteria was considerably less than that of unwashed bacteria. As shown in Table V, the titer was decreased and the intensity of the reaction outside the zone of granular agglutination was markedly less with the former. The above observations point to the removal of a certain number of flagella from the bacteria with each washing manipulation. Upon centrifuging, the bacteria are sedimented while the flagella remain free in the supernatant. With the addition of specific antiserum flagella and agglutinin unite and a floccular agglutination results.

DISCUSSION

The preceding observations have proved readily reproducible with the technic outlined. An interpretation linking them together and offering a possible explanation of the described anomaly is presented:—

The resuspension of sedimented motile bacteria in a fluid medium results in the mechanical removal of some of the attached flagella. If the bacteria are unsensitized the flagella remain in suspension after centrifugation, and the clear supernatant gives a typical flagellar agglutination upon the addition of specific antiserum. If the bacteria are sensitized the free flagella, which are in combination with agglutinin, clump in the presence of salt and are removed upon centrifugation. The initial water washing of sensitized motile bacteria contains suffi-

cient salt from the previous absorption mixture to cause clumping of the flagella. The clumped flagella are removed by sedimentation and consequently the supernatant is inactive, failing to flocculate upon the addition of saline. The subsequent water washings are salt-free and the freed flagella, either with attached or freed agglutinin, remain in suspension. The water washings of sensitized bacteria are cloudy at this stage owing possibly to minute aggregates of flagella which are too small for sedimentation.

That agglutinin in some form is removed from flagella in the salt-free medium seems apparent. Nothing definite can be said concerning its physical state other than the fact that it is again able to unite with whole bacteria, causing a clear-cut agglutination in moderate dilution.

The water washings of sensitized bacteria, at this stage, also flocculate upon the addition of saline alone. The flocculation is in reality a flagellar agglutination which was previously inhibited by the absence of salt. In the presence of salt, recombination of flagella and agglutinin, or union of flagella already in combination with agglutinin, occurs, with subsequent clumping.

It is maintained that the floccular reaction which results when the salt-free water washings are mixed with a saline suspension of heated bacteria is in reality a flagellar agglutination and is entirely independent of the added antigen. Flagella and flagellar agglutinin are both present in the salt-free washing fluids. Salt is supplied by the added bacterial suspension (heated) and flagellar agglutination results. In the presence of whole bacteria agglutinin may be diverted from the free flagella by the excess of those attached to the bacteria causing under such circumstances a bacterial agglutination.

As regards the differential removal of agglutinin it may be said that flagellar agglutinin in a state capable of again causing flocculation of whole bacteria may be freed from sensitized motile bacteria by washing with salt-free water. With the degree of sensitization brought about in the work here reported somatic agglutinin, on the other hand, is not demonstrable in the washings.

SUMMARY

The salt-free water washings of a sensitized motile bacterium (*B. aertrycke*) were found to cause a floccular agglutination in the

presence of both whole and deflagellated antigen. Evidence was presented that the water washings when salt-free contained flagella and flagellar agglutinin and that clumping occurred upon the addition of saline. The floccular reaction in the presence of deflagellated bacteria was regarded as the agglutination of flagella present in the washings. In the presence of whole bacteria, however, actual bacterial agglutination resulted.

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