THE EFFECT ON SUBSEQUENT AGGLUTINATION OF THE EXPOSURE OF BACTERIA TO HEATED ANTISERUM.

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(Received for publication, November 9, 1927.)

Eisenberg and Volk (1) have shown that bacteria treated for intervals of 1 to 2 hours with immune serum heated to 65°C. or 70°C. failed to agglutinate when the heated serum was removed and fresh immune serum added. Ehrlich's conception is that agglutinin is composed of two substances, one that brings about the combination of cell and antibody and another that produces agglutination. His explanation of Eisenberg and Volk's phenomenon assumes that the zymophore substance is changed by heat into agglutinoid which no longer produces clumping. On the other hand, the haptophore portion is unaltered and still combines with the cell to such an extent that further union cannot take place on the addition of unaltered immune serum. This has been accepted as the explanation of the phenomenon.

It seemed possible that Eisenberg and Volk's phenomenon might be explained in other ways. Any serum when heated and added to a bacterial suspension might stick to the surface of the bacterial cell and thus prevent specific union on the subsequent addition of unheated immune serum. Furthermore, it is necessary to show by experiment that the agglutinin or antibody has been completely destroyed when the serum is heated in order to explain the process on the basis of agglutinoid combination. This is especially true in the light of the observations of Beyer and Reagh (2), Orcutt (3), and F. S. Jones (4) that certain types of agglutinin are not destroyed when heated at 70°C. for 20 minutes. A further suggestion is that when too concentrated heated serum is added the reaction may not take place because

of an excess of protein. To answer the questions thus brought up a series of experiments was undertaken.

EXPERIMENTAL.

The agglutinin was prepared by the immunization of rabbits with a motile strain of the hog cholera bacillus and *Bacillus abortus*. The agglutinating serum had been stored in the refrigerator for a month or more. Details of procedure are recorded under the separate experiments.

Experiment 1.—The growth from two 24 hour agar slants of the motile hog cholera bacillus was suspended in 2 cc. NaCl solution. Amounts of 0.3 cc. of this suspension were added to 1.5 cc. of a 1:1 dilution of hog cholera bacillus antiserum which had been heated at 75°C. for 30 minutes; to normal rabbit serum diluted 1:1 and also heated at 75°C.; to normal rabbit serum diluted 1:1; and to 2 cc. of cow serum heated at 65°C. for ½ hour. For control purposes the same amount of culture suspension was added to 1.5 cc. of NaCl solution. All tubes were incubated 1½ hours, then 8 cc. of sterile salt solution was added and the tubes centrifuged rapidly. The supernatant liquid was withdrawn and the bacilli resuspended in 10 cc. of salt solution. The suspensions were then tested with hog cholera bacillus serum. Readings were made after 2 hours incubation and refrigeration overnight.

This experiment was repeated with similar results. From the protocol submitted it is evident that exposure to normal serum fails to appreciably affect the agglutinability of the bacilli. However, when the culture was added to the specific serum which had first been heated, the addition of further agglutinin did not materially increase the subsequent agglutination. It is well known from the work of others that 75°C. does not completely destroy the flagellar agglutinin, and as indicated in the control tube, some agglutination had already taken place. During the exposure the contents of each tube were repeatedly agitated by drawing up and rapidly expelling the mixture with capillary pipettes, so that any clumps were readily broken. In addition, after centrifugation the bacilli in the first series were difficult to resuspend and the suspension was relatively unstable since deposition occurred throughout the tubes of the series. It is possible that a little agglutinin still remaining in the serum combines with the bacterial cells, but through mechanical means the clumps may be largely broken so that the bacterial suspension no longer reacts markedly with its antiserum.

It will be noted in the first experiment that heating agglutinin to 75°C. confirmed in a large measure the findings of Eisenberg and Volk. Their experiments were conducted after heating the agglutinin to 65°C. or 70°C. When the lower temperatures were tried with the hog cholera bacillus agglutinin the bacilli were promptly agglutinated, and although the suspensions were agitated vigorously the bacilli settled to the bottom of the tubes after resuspension in salt solution.

It seemed of further interest to observe the effect on subsequent

TABLE I.

The Effect on Subsequent Agglutination of Exposing the Hog Cholera Bacillus to Various Sera.

	Dilutions of unheated hog cholera bacillus immune serum									
	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	Control
Bacilli exposed to hog cholera bacillus antiserum diluted 1:1 and heated at 75°C. for ½ hr.	++	++	++	++	++	+	+	+	+	+
Bacilli exposed to heated rabbit serum	C	C	C	C	C	+++	+	−	−	−
Bacilli exposed to unheated rabbit serum	C	C	C	C	++	++	−	−	−	−
Bacilli exposed to heated cow serum	C	C	C	C	C	+++	+	±	−	−
Bacilli exposed to NaCl solution	C	C	C	C	C	+++	++	+	±	−

Agglutination has been reported as follows: C, the maximum, with heavy deposit and complete clearing; ++++, not quite complete; +++, strong clumping; ++, well defined clumping and a definite deposit in the bottom of the tube; ±, a slight deposit.

agglutination of exposure of the bacilli to immune serum heated at higher temperatures. Experiment 2 covers this phase of the question.

Experiment 2.—The immune and normal rabbit sera were each diluted in 4 parts of salt solution. A portion of each was heated at 75°C. for 20 minutes, another lot was exposed to 80°C. for the same period. To each 2.5 cc. of the heated diluted serum, 0.3 cc. of a heavy suspension of living hog cholera bacilli was added. The same amount of culture was added to NaCl solution for control purposes. All tubes were incubated 1 hour and the contents mixed repeatedly with capillary pipettes. After incubation, 8 cc. of NaCl was added and the mixture centrifuged. The supernatant was then poured off and the bacilli resuspended in 10 cc. of salt

solution. The suspensions were then tested with immune serum. The findings are given in Table II.

It will be noted that the data submitted in the second experiment confirm the first observation. When the bacilli are first submitted to antiserum which has been heated at 75°C. for 30 minutes, they fail to agglutinate to any great extent on the addition of fresh agglutinin. When the immune serum is heated at 80°C. for 30 minutes, the effect is less marked although the agglutinin titer of the unheated serum is appreciably diminished. The effect cannot be ascribed to mechanical

TABLE II.

The Effect of Exposing the Hog Cholera Bacillus to Serum Heated at 75°C. and 80°C.

	Dilutions of hog cholera bacillus immune serum									
	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	Control
Bacilli exposed to hog cholera bacillus antiserum diluted 1:4 and heated at 75°C. for ½ hr.	++	+	±	±	±	±	−	−	−	−
Bacilli exposed to hog cholera bacillus serum diluted 1:4 and heated at 80°C. for ½ hr.	C	C	C	++++	+++	++	−	−	−	−
Bacilli exposed to normal rabbit serum diluted 1:4 and heated for ½ hr. at										
75°C.	C	C	C	C	C	++++	+	±	−	−
80°C.	C	C	C	C	C	++++	+	−	−	−
Bacilli exposed to NaCl for the same period	C	C	C	C	C	++++	+	−	−	−

influences of serum or slowing down of motility, since the organisms exposed to heated rabbit serum or salt solution continued to agglutinate well with the immune serum. Data not given in the table show that immune serum heated at 90°C. for 30 minutes has no appreciable influence on subsequent agglutination.

It seemed of further interest to ascertain to what extent the proteins of the immune and normal rabbit sera actually combined with the bacterial cells. The writer (5) had previously shown that collodion particles and in certain cases bacteria bathed in solutions of various proteins retained sufficient on their surfaces so that they were agglu-

tinated on the addition of precipitin specific for the sensitizing protein. With a similar procedure it was hoped that actual combination could be shown.

Experiment 3.—As in the previous experiment, the immune and normal rabbit sera were diluted 1:4. A portion of each was heated at 75°C. and 80°C. for ½ hour. To 3 cc. portions of diluted serum 0.3 cc. of bacterial suspension was added. They were then incubated 1 hour, an excess of salt solution was added, and the tubes centrifuged. The bacilli were then washed twice more in salt solution and finally resuspended in 10 cc. NaCl. The resuspensions were tested with anti-rabbit serum precipitin prepared by injecting a fowl with rabbit serum. The results are given in Table III.

TABLE III.

The Effect of Rabbit Serum Precipitin on Hog Cholera Bacilli First Exposed to Heated Sera.

	Dilutions of rabbit serum precipitin							
	1:50	1:100	1:200	1:500	1:1,000	1:2,000	1:5,000	Control
Culture first treated with antiserum heated at 75°C. for 20 min.	++++	++++	+++	++	+	+	+	±
Culture first treated with antiserum heated at 80°C. for 20 min.	++++	++	+	±	±	−	−	−
Culture first treated with normal rabbit serum heated at 75°C. for 20 min.	++	±	−	−	−	−	−	−
Culture first treated with normal rabbit serum heated at 80°C. for 20 min.	++	±	−	−	−	−	−	−
Culture carried in NaCl solution	±	−	−	−	−	−	−	−

From the protocol submitted in Table III, it is evident that when immune serum containing antibody has been heated at 75°C. for 20 minutes, certain of the antibodies are still capable of combining with the bacterial cell and such combination may be detected by precipitin. Thus during union of bacillus and antibody certain of the serum proteins are deposited on the bacteria, and this protein deposit will react with its specific antibody and agglutination result. That there had been considerable deposition of serum protein on the bacilli exposed to the serum heated at 75°C. is clear. It is also true that serum heated at 80°C. is not as efficient in this regard, but nevertheless considerable

must be deposited on the bacterial cell surfaces. The reaction in this instance is well defined, since agglutination occurs when as little as 1/200 cc. of precipitin is added. The control series in which the bacilli were acted upon by normal serum show only slight fixation between bacterium and rabbit serum protein. The bacilli carried in salt solution, but otherwise manipulated in a similar manner, failed to agglutinate when treated with the rabbit serum precipitin.

As a further confirmation of the preceding experiments the following experiment may be briefly cited.

Experiment 4.—When 0.5 cc. of bacillary suspension is added to a suitable amount of complement and 0.5 cc. of antiserum diluted 1:4, and, after suitable incubation, amboceptor and red cells are added, no hemolysis results. The same holds true when the diluted antisera are heated for 20 minutes at 70°C. and 75°C. When the antiserum has been heated to 80°C. there is still considerable diversion of complement (a ++ reaction). When the antiserum is heated to 90°C. even less of the complement is diverted although the hemolysis is not complete.

As the experiment shows, there remains in the serum heated at 70°, 75°, and 80°C. an antibody still capable of combining with its antigen in sufficient quantities to entirely or partially divert complement.

When another organism and its specific serum are substituted for the hog cholera bacillus, essentially the same results are obtained, as will be brought out in Experiment 5.

Experiment 5.—Normal rabbit serum and rabbit serum containing agglutinin for *B. abortus* were diluted in 4 parts of NaCl solution. The diluted serum was distributed in amounts of 3 cc. in a series of tubes, and the tubes were then heated at 70°, 75°, 80°, 85°, and 90°C. for 20 minute intervals. A heavy suspension of *B. abortus* was added to each tube and all were incubated 1½ hours. A tube of bacterial suspension in salt solution was also incubated. The contents of all tubes were agitated at frequent intervals. After incubation, an excess of salt solution was added and the tubes centrifuged. The bacteria were then resuspended in salt solution and tested with unheated specific serum. The results of this treatment on subsequent agglutination are given in Table IV. The controls, where normal rabbit serum was used, failed to show that such treatment influenced subsequent agglutination and are for this reason omitted from the table.

The *Bacillus abortus* serum on the whole behaves when heated much like the other agglutinin. There is however this difference, that the

substance in the abortion bacillus antiserum is more resistant to heat since sufficient combination existed even after heating the antiserum to 80°C. to prevent agglutination on addition of unheated immune serum. Immune sera heated to 85°C. and 90°C. have no inhibiting effect.

That there is an actual fixation between bacterial cell and serum protein can again be shown by noting the effect when rabbit serum precipitin is added to the bacterial suspension which had first been treated with heated agglutinin. This has been brought out in Experiment 6.

TABLE IV.

The Behavior of Bacillus abortus after Treatment with Heated Immune Serum.

	Dilutions of unheated immune rabbit serum							
	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	Control
Bacilli exposed to diluted serum heated at °C.								
70	—	—	—	—	—	—	—	—
75	—	—	—	—	—	—	—	—
80	—	—	—	—	—	—	—	—
85	C	C	++++	+++	++	+	+	—
90	C	C	++++	+++	++	+	+	—
Control—bacilli exposed to NaCl	C	C	C	C	++	+	+	—

Experiment 6.—This experiment is virtually a repetition of Experiment 3 except that *B. abortus* and its agglutinin were used. The procedure was the same. The results are given in Table V.

As in Experiment 3, Table V indicates that antiserum heated at 75°C. actually combines with the bacterial cell in such quantities that agglutination results when a precipitin specific for rabbit serum is added. The blood serum proteins are not dislodged by washing. The combination is less marked when the immune serum is first heated at 80°C., nevertheless the reaction is more intense than that occurring after suspension in immune serum heated at 85°C. and 90°C., or normal rabbit serum heated at the same temperatures.

It was possible by complement fixation tests to further confirm the findings that immune serum heated to 75°C. for 20 minutes completely

deviates the complement in the presence of antigen. That heated to 80°C. markedly deviates the complement, while there is only a slight deviation with serum heated at 85°C., and none with that heated at 90°C.

DISCUSSION.

It is true that bacteria when first treated with immune serum heated at various temperatures may or may not agglutinate on subsequent

TABLE V.

The Effect of Rabbit Serum Precipitin on Bacillus abortus First Treated with Heated Immune Serum.

	Cc. of rabbit serum precipitin					
	1:50	1:100	1:200	1:500	1:1,000	Control
Bacilli treated 1½ hr. with immune serum diluted 1:4 first heated for 20 min. at °C.						
75	+++	++++	++	+	+	−
80	+++	++	−	−	−	−
85	+	+	−	−	−	−
90	+	±	−	−	−	−
Bacilli treated with diluted normal rabbit serum first heated at 75°, 80°, 85°, and 90°C.	+	±	−	−	−	−
Bacilli treated with NaCl solution	−	−	−	−	−	−

exposure to agglutinin. When the hog cholera bacillus antiserum was heated to 75°C. for 20 minutes, the bacilli exposed to its action failed to agglutinate when subsequently treated with unheated immune serum. When the first test serum is heated at 80°C., relatively little inhibiting effect is encountered. The reaction is a specific one, since it is not encountered when normal serum is used in a similar manner. In this regard *Bacillus abortus* acts much like the hog cholera bacillus when similar experiments are performed. However, *Bacillus abortus* serum heated at 80°C. is still capable of preventing further agglutination of the bacilli.

Ehrlich's conception is that two substances produce agglutination,—one is a combining body which is thermostabile, and the other produces clumping and is thermolabile. The latter is converted into agglutinoid

by heat. The combining substance is uninjured by heat and enters into combination with the bacterial cell, thus preventing union on the addition of fresh agglutinin. The evidence that I have submitted while not conclusive is at least suggestive that the reaction may be explained on other grounds.

In the first place, the temperature at which the earlier investigators heated their sera (65°C. and 70°C.) has in my hands always agglutinated the organisms. Furthermore, they worked with undiluted serum and it is possible that agglutination failed to take place because of an excess of colloid, *i.e.*, they may have been working in the prozone. When the evidence in regard to the hog cholera bacillus and its agglutinin is analyzed more carefully it is evident that heating at 75°C. for 20 minutes is insufficient to destroy the agglutinin. In fact, the bacilli will agglutinate with such serum provided the incubation is long enough and if interfering mechanical factors are avoided. It has been shown that this is the case by several investigators. The writer (4) brought out the fact that as the temperature increases the agglutinin content declines, at first gradually, until 75°C. is reached. The break is sharp at 80°C., although a little antibody can be detected even after heating to 90°C. There remains, then, in the case of this serum considerable agglutinin after heating the serum to 75°C. It is suggested that this agglutinin combines with the bacterial cell, but as the result of mixing, too short incubation, or for other reasons, the combination is not quantitatively sufficient to produce the phenomenon of agglutination. It may be that agglutinin is so modified during heating at certain temperatures that, although still capable of combining with the organisms, the usual phenomenon of such combination (agglutination) fails to take place. The union is apparently sufficient to prevent further union when fresh immune serum is added. That union actually takes place between the bacterial cell and the serum proteins of certain heated sera is definitely shown by the reaction when a specific precipitin is added, since it is known that agglutination results when bacteria or collodion particles are sensitized to proteins on addition of specific precipitin. As further evidence the behavior of the heated serum in the complement fixation tests is suggestive. In both cases cited the sera heated sufficiently to prevent the secondary agglutination after the addition of fresh serum always deviated the complement. When the sera were heated to higher

temperatures and failed to prevent the secondary agglutination, then relatively little or none of the complement was deviated.

The evidence for *Bacillus abortus* agglutinin is not so clear, although it is known that the agglutinin will resist heating to 70°C. for 20 minutes. Serum heated above this point no longer causes agglutination. Nevertheless when the immune serum is heated to 75°C. and 80°C. it is still capable of preventing a second agglutination. It is clear, however, that actual union of bacterial cell and serum proteins occurs even after heating the immune serum to these temperatures. It is also true that such sera were able to bind complement.

In the light of the experimental evidence the burden of proof that agglutinoid actually exists remains to be established, since the blocking of secondary agglutination can be explained on the basis of residual antibody.

SUMMARY.

It is shown that when dilute rabbit serum rich in agglutinin for the hog cholera bacillus is heated at 75°C. for 20 minutes and the bacilli incubated with the heated serum, agglutination fails to result on the addition of unheated immune serum. When the immune serum is first heated to 80°C., it no longer greatly inhibits secondary agglutination when the organisms are exposed to fresh agglutinin. The abortion bacillus agglutinin acts in a similar manner except that the immune serum must be heated above 80°C. for 20 minutes to prevent the second agglutination. The reactions are specific since control experiments with normal rabbit serum heated at various temperatures failed to influence further agglutination. It has also been shown by precipitation tests that there is definite fixation of serum proteins and bacterial cells with the heated sera which would prevent subsequent agglutination. Furthermore, heated antiserum which would prevent the secondary agglutination still possessed the property of deviating complement in a hemolytic series.

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