THE RELATION OF CERTAIN BACTERIA TO NON SPECIFIC REACTIONS WITH THE COMPLEMENT FIXATION TEST FOR LUES.*

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Several investigators who have had a large experience with the complement fixation test for lues have called attention to the development in sera of anticomplementary bodies which cause inhibition of hemolysis in both the antigen and control tubes, and they have found that in most instances this property of the serum is lost after heating it for half an hour at 56° C. In some instances, however, the property still persists after heating, and in such sera one is unable to read the reaction. It would appear from these observations that the anticomplementary bodies are of two kinds, thermolabile and thermostabile, and that the inactivation of serum enables us to destroy only the thermolabile group.

The exact nature of these anticomplementary substances is still in doubt, but it is the purpose of this contribution to show that certain strains of bacteria, if growing in human blood serum, are capable of producing total inhibition of hemolysis in both antigen and control tubes; and that not only is this true, but certain bacteria also possess the power of producing inhibition in the antigen tubes alone, thus giving rise to a non-specific reaction in the sera which contain them.

In the application of the complement fixation to the diagnosis of lues in the military service, we have adopted in this laboratory the modification of the Wassermann test devised by Noguchi, the only departure from his technique being the use of an alcoholic extract of fetal luetic liver as antigen, and the inactivation of the serum

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before testing. As we make tests for many army posts at some distance from this laboratory, the use of active serum is impossible, as most of the serum reaching us is at least two, and often from three to four days old. For this reason, and in order to avoid the thermolabile anticomplementary bodies and the non-specific reactions sometimes observed when an alcoholic extract is used as antigen, the sera are always inactivated just before making the test. The results we have obtained in over 2,000 complement fixation tests have justified the technique employed, and have shown that, with the Noguchi modification of the Wassermann test, it is possible to use sera several days old with as good results as fresh sera, provided that bacterial contamination is avoided, and that the sera are inactivated before the test is made. Serum in which hemolysis has occurred can not be used on account of the difficulty of reading the reaction; so it is important that the serum be allowed to separate thoroughly from the clot before it is sent to the laboratory.

Before adopting the Noguchi modification of the complement fixation test in the army, we conducted careful experiments upon the keeping properties of human sera, especially as some authorities had reported that certain specimens of normal serum, if kept for several days, would give a positive reaction. Our experiments convince us that as long as normal sera remain free from bacteria they do not give a positive reaction, even though kept at room temperature for a month, although in some of the sera experimented with, both thermolabile and thermostabile anticomplementary bodies developed, which caused inhibition of hemolysis in both antigen and control tubes.

In all, fifty normal sera were tested, the blood being collected in Wright tubes and the serum allowed to separate, after which it was pipetted into suitable glass containers and sealed. Enough of these tubes were prepared to enable us to make a complement fixation test upon each serum at intervals of one week for a month. Of the fifty sera examined, all gave a negative reaction at the time of collection and forty-three were still negative at the end of a month, while seven had developed anticomplementary bodies which produced complete inhibition of hemolysis in both antigen and control tubes. As an alcoholic extract was used as antigen, the sera were

always inactivated before the test was made, but in two sera tested before inactivation, both being about two days old, a positive reaction was obtained. Both of these sera gave a negative reaction after inactivation, and neither was contaminated by bacteria. This observation confirms that of other investigators who have found that non-specific reactions may occur in testing active sera when an alcoholic extract is used as antigen.

Our results in this series of experiments convince us that a positive reaction does not occur in normal sera for a period of time far in excess of any delay that may occur before the specimens reach the laboratory, provided that proper care is taken in collecting them, and that the sera are inactivated before use. The following table (table I) gives the results obtained in one series of experiments and is illustrative of all with normal sera free from bacterial contamination.

TABLE I.

*Complement Fixation in Normal Sera, Kept at Room Temperature for One Month.**

No. of serum.	Date.	Result.	Date.	Result.	Date.	Result.	Date.	Result.
1	Jan. 13	—	Jan. 21	—	Jan. 28	—	Feb. 4	—
2	"	—	"	—	"	—	"	—
3	"	—	"	—	"	—	"	—
4	"	—	"	—	"	—	"	—
5	"	—	"	—	"	—	"	—
6	"	—	"	—	"	—	"	—
7	"	—	"	—	"	—	"	—
8	"	—	"	—	"	—	"	—

* The blood was collected in Wright tubes. The serum was allowed to separate and was then transferred to glass tubes and sealed, one tube being opened for each test.

The importance of the prevention of bacterial contamination of the sera was recently emphasized in the case of a specimen sent from San Francisco to this laboratory during some experiments we were making upon the effect of transportation on serum submitted for the complement fixation test. Arrangements were made with the laboratory of the U. S. Army General Hospital at San Francisco, California, to send us each week duplicates of sera that were tested there, in order that we might repeat the tests and thus ascertain whether any changes had occurred in the sera during trans-

portation. All the sera sent us from San Francisco were at least six days old when received, and the following table (table II) gives the results obtained in both laboratories.

TABLE II.

Results of Comparative Complement Fixation Tests upon 80 Sera at San Francisco and Washington, D.C.

Case no.	Test 1.		Case no.	Test 1.		Test 2.		Test 3.		Test 4.	
	S. F.*	Wash.*		S. F.	Wash.	S. F.	Wash.	S. F.	Wash.	S. F.	Wash.
1	−	−	41	?	?						
2	−	−	42	−	−						
3	+	+	43	?	?						
4	+	+	44	+	+						
5	−	−	45	+	+						
6	−	−	46	−	−						
7	+	+	47	−	−						
8	−	−	48	+	+						
9	−	−	49	−	−						
10	+	+	50	−	−						
11	+	+	51	+	+						
12	+	+	52	+	+	+	+	+	+	+	+
13	+	+	53	−	−	−	−	−	−		
14	−	+	54	+	+	+	+	+	+		
15	−	−	55	+	+	+	+	+	+		
16	+	+	56	−	−	−	−				
17	−	−	57	+	+	+	+				
18	−	−	58	+	+	+	+				
19	−	−	59	−	−	−	−				
20	−	−	60	+	+	+	+				
21	−	−	61	+	+	+	+				
22	−	−	62	−	−	−	−				
23	+	+	63	−	−	−	−				
24	+	+	64	+	+	+	+				
25	−	−	65	+	+	+	+				
26	−	−	66	−	−	−	−				
27	−	−	67	+	+	+	+				
28	−	−	68	+	o						
29	−	−	69	+	o						
30	+	+	70	+	o						
31	−	−	71	+	o						
32	−	−	72	+	o						
33	−	−	73	−	o						
34	+	+	74	+	o						
35	+	+	75	+	o						
36	−	−	76	+	o						
37	−	−	77	+	o						
38	+	+	78	−	o						
39	+	+	79	+	o						
40	+	+	80	+	o						

* S. F. = San Francisco. Wash. = Washington, D. C.

A consideration of the table shows that in sixty-seven of the eighty sera recorded the results were identical except in one in-

stance, a serum which gave a negative result at San Francisco and a positive one in this laboratory. There were thirteen sera in which no result could be obtained in this laboratory because of the development of thermostabile anticomplementary bodies during the interval of time elapsing between the test in San Francisco and that in Washington. In all of these sera, inhibition of hemolysis occurred in both antigen and control tubes, and all were contaminated with bacteria.

The occurrence of a positive result in one specimen of serum which was negative at San Francisco suggested that perhaps certain bacterial substances might have developed in the serum which gave a positive reaction, especially as it appeared cloudy and had a putrefactive odor. In order to determine this point it was plated out upon agar-agar and pure cultures of the following organisms were finally obtained: *Staphylococcus aureus;* a large *Staphylococcus albus;* a small *Staphylococcus albus;* a large spore-bearing bacillus of the subtilis type; and a very minute diplobacillus.

The following complement fixation experiments were undertaken with these organisms, and also with a stock culture of *Staphylococcus aureus, Staphylococcus citreus,* and *Streptococcus pyogenes.* The same technique was employed in these experiments as in the routine complement fixation tests made in the laboratory, the mixtures of serum and bacteria being substituted for pure serum.

TABLE III.

Result of Complement Fixation Tests in Experiment I.

Name of bacterium.	Result.		Controls.		Origin of Bacterium.
	Antigen tube.	Control tube.	Bacteria alone.	Serum alone.	
S. albus (large)	—	—	—	—	Isolated from serum.
S. albus (small)	—	—	—	—	" " "
S. aureus	—	—	—	—	" " "
Bacillus (spore)	—	—	—	—	" " "
Diplobacillus	—	—	—	—	" " "
Combined bacteria.	—	—	—	—	" " "

Experiment 1.—An emulsion in normal salt solution was made from a pure culture of each organism isolated from the serum, and four drops of the emulsion were placed in 1 c.c. of a 1 per cent. suspension of human red blood corpuscles, together with four drops of an inactivated normal serum. An emulsion was also made of all the bacteria combined, and four drops of this and four drops of

normal serum were added to the same amount of blood suspension. Table III gives the results obtained in this experiment.

From the results obtained in this experiment it is evident that merely adding an emulsion of the cultures of the bacteria to normal serum is without effect upon the result of the complement fixation test, as all of the mixtures gave a negative reaction.

Experiment 2.—This was identical with experiment I except that the mixtures of serum and bacteria were inactivated at 56° C. for one half hour before testing. The results are given in table IV.

TABLE IV.

Result of Complement Fixation Tests in Experiment 2.

Name of bacterium.	Result.		Controls.		Origin of bacterium.
	Antigen tube.	Control tube.	Bacteria alone.	Serum alone.	
S. albus (large)	−	−	−	−	Isolated from serum.
S. albus (small)	−	−	−	−	" " "
S. aureus	−	−	−	−	" " "
Bacillus (spore)	−	−	−	−	" " "
Diplobacillus	+	+	+	−	" " "
Combined bacteria	−	−	−	−	" " "

It would appear from this experiment that the heating of the mixture of serum and the diplobacillus resulted in the liberation of inhibitory substances sufficient to cause inhibition of hemolysis in both the antigen and control tubes. All the other mixtures gave a negative result.

Experiment 3.—Specimens of normal serum were inoculated with each of the bacteria isolated from the serum as well as with a stock culture of *S. aureus, S. citreus,* and *Streptococcus pyogenes,* and allowed to stand at room temperature for one week before testing. Table V gives the result of this experiment.

TABLE V.

Result of Complement Fixation Tests in Experiment 3.

Name of bacterium.	Result.		Control.	Origin of bacterium.
	Antigen tube.	Control tube.	Serum alone.	
S. albus (large)	+−	−	−	Isolated from serum.
S. albus (small)	−	−	−	" " "
S. aureus	−	−	−	" " "
Bacillus (spore)	−	−	−	" " "
Diplobacillus	+	++	−	" " "
S. aureus	++	−	−	Stock culture.
S. citreus	++	++	−	" "
S. pyogenes	−	−	−	" "

It is evident from this experiment that after incubation in human serum for one week at room temperature the large *S. albus* isolated from the serum under discussion gave a plus-minus reaction in the antigen tube, while the diplobacillus isolated from the same serum gave a plus reaction in the antigen tube, and a double plus reaction in the control tube. The stock culture of *S. aureus* gave a double plus positive reaction, while the stock culture of *S. citreus* caused total inhibition in both antigen and control tubes.

Experiment 4.—This was identical with experiment 3, except that the mixtures of serum and bacteria were incubated for twenty-four hours at 37° C. and then allowed to stand at room temperature for one week. Table VI gives the results of this experiment.

TABLE VI.

Result of Complement Fixation Tests in Experiment 4.

Name of bacterium.	Result.		Control.	Origin of bacterium.
	Antigen tube.	Control tube.	Serum alone.	
S. albus (large)	++	−	−	Isolated from serum.
S. albus (small)	−	−	−	" " "
S. aureus	++	−	−	" " "
Bacillus (spore)	−	−	−	" " "
Diplobacillus	++	++	−	" " "
S. aureus	++	−	−	Stock culture.
S. citreus	++	++	−	" "
S. pyogenes	++	−	−	" "

The results were surprising in that no less than four of the organisms tested gave a double plus positive reaction in the normal serum alone, while two inhibited hemolysis in both antigen and control tubes. It would appear that the twenty-four hours at incubator temperature lead to an increase in the intensity of the reaction, probably by favoring a richer development of the bacteria in the sera.

Considering the experiments as a whole, it is perfectly evident that a positive complement fixation reaction may be obtained in a normal serum if certain species of bacteria are enabled to develop within it. That every strain of a certain bacterial species will not produce this result is shown by the fact that while a double plus reaction occurred in the serum infected with the stock *S. aureus* and with the *S. aureus* isolated from the serum under discussion, a negative result was obtained with another *S. aureus* isolated from a serum which gave a negative reaction.

It is also evident that temperature has much to do with the ability of the bacteria to produce a non-specific reaction, for in the mixtures of serum and bacteria kept for twenty-four hours at incubator temperature, the reactions were most marked, while two of the organisms which did not give a reaction when growing in the serum at room temperature, gave strong positive reactions when incubated at 37° C. for twenty-four hours. Inactivation also appears to favor the production of a false positive reaction in sera which are contaminated with certain bacteria. As regards the San Francisco serum under discussion, it is evident that the positive result at this laboratory could have been caused by either the large *S. albus* or the *S. aureus,* or by both, according as the conditions at the time of the examination were favorable to the production of inhibitory substances by one or both of these organisms. The following is a summary of the action of each of the bacteria under the conditions of the various experiments.

The large *S. albus* isolated from the serum under discussion gave a negative result in both antigen and control tubes when added directly to normal serum, whether the mixture was or was not inactivated; when it was added to normal serum and kept at room temperature for a week and inactivated, it gave a plus-minus positive reaction; and finally, when added to normal serum, kept at incubator temperature for twenty-four hours and at room temperature for one week, the mixture being then inactivated, a double plus positive reaction was obtained. This organism was the most consistent in its behavior toward the complement fixation test of any of those examined, and its activity in giving a positive reaction seems to depend entirely upon conditions favorable to its growth in the serum.

The small *S. albus* from the same serum gave a negative result in both tubes under every experimental condition.

The *S. aureus* which was isolated from the serum, gave a negative result in both tubes under all conditions except those of experiment 4, in which it gave a double plus positive reaction. It is undoubtedly true that the more favorable conditions for growth in this experiment account for the result obtained.

The large spore-bearing bacillus gave a negative result in both tubes throughout the experiments.

The small diplobacillus inhibited hemolysis in both tubes except in experiment 1, where it gave a negative result in both tubes.

The stock *S. aureus,* under the conditions of experiments 3 and 4, the only ones in which it was tried, gave a double plus positive reaction.

The stock *S. citreus,* under the conditions of experiments 3 and 4, the only ones in which it was tried, gave complete inhibition of hemolysis in both tubes.

The stock *Streptococcus pyogenes,* under the conditions of experiment 4, gave a double plus positive result, but a negative result under the conditions of experiment 3. Here again the result, as regards a positive reaction, appeared to depend upon favorable cultural conditions.

Under the conditions of experiment 4, which were apparently the most favorable to the production of a positive result in the mixtures of bacteria and serum, an *S. aureus* isolated from a negative serum failed to give a positive result, so that it is evident that certain strains of the same species differ in this respect.

The fact that under certain conditions such common bacteria as staphylococci and streptococci, when growing in normal serum, may give rise to a positive Wassermann reaction, is of the greatest practical importance, and one might conclude from these experiments that only fresh sera should be used in making complement fixation tests; but if proper precautions are taken in the collection of the specimens, there is no danger of bacterial contamination, and we believe that, unless such contamination occurs, a positive result will not be obtained in a normal serum. Our experiments have shown that sera may be kept at room temperature for as long as a month without danger of a negative serum becoming positive, and we have observed such a phenomenon only in sera contaminated with bacteria. Moreover, contaminated sera may be easily detected, as they appear cloudy and have a disagreeable odor.

However, these observations indicate the necessity of asepsis in the collection of blood for the Wassermann test, if the test can not be made within twenty-four hours after collection, and we believe that such precautions should always be taken in the collection of blood for this purpose. The needle or lancet used for puncture,

and the Wright tube in which the blood is collected, should be sterilized, and the skin over the site of puncture thoroughly washed with alcohol. When the blood is to be examined at once, such measures are unnecessary, but they should always be adopted if the serum is to be kept twenty-four hours or longer before making the test.

We believe that the results obtained in the experiments described justify the following conclusions:

1. Certain strains of staphylococci and streptococci, when growing under favorable conditions in normal human blood serum, are capable of producing substances in the serum which cause a positive result with the complement fixation test for lues.

2. These non-specific reactions do not occur in normal serum which is uncontaminated with bacteria, even though the serum be kept at room temperature for one month.

3. The intensity of the positive reaction in contaminated sera apparently depends upon the presence of favorable cultural conditions for the bacteria concerned, as contaminated sera in which the bacteria can not develop do not give a positive reaction.

4. When growing in normal blood serum certain strains of staphylococci and saprophytic bacilli produce thermostabile anticomplementary bodies which are capable of causing total inhibition of hemolysis in both the antigen and control tubes.

5. Non-specific reactions due to bacteria are probably very rare in practice, but they are of sufficient importance to justify the use of aseptic methods in the collection of blood for the complement fixation test.