An important new development in the serodiagnosis of syphilis is the demonstration of at least two Treponema pallidum agglutinating antibodies in syphilitic serums.

Agglutination of Treponema pallidum by Reagin Antibody

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SPECIFIC AGGLUTINATION of killed *Treponema pallidum* has been demonstrated by several investigators (*1–3*). Recently, McLeod and Magnuson (*4*) showed that agglutination of *T. pallidum* in syphilitic serum was greatly enhanced by the conglutinating action (*5*) of fresh steer serum. A preliminary evaluation (*4*) of this technique as a diagnostic test for syphilis indicated that the comparatively simple agglutination test might be as sensitive and specific as the *T. pallidum* immobilization (TPI) test (*6*). Subsequent experiments, however, have made clear that the agglutination test in its present form detects more than one antibody and is in part a measure of reagin. These investigations, together with a study of certain factors which influence the sensitivity of the test, are the subject of the present paper.

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Methods

The methods used were similar to those earlier described in detail (*4*). Spirochetes were extracted in saline from testicular lesions of rabbits inoculated 7 to 10 days earlier with the Nichols strain of *T. pallidum*. The organisms were sedimented by centrifugation, resuspended in fresh saline, and the suspensions adjusted to contain approximately 60 to 75 spirochetes per high-power field. In order to insure even distribution of the organisms, bovine albumin fraction V was added in a final concentration of 5 percent. The antigens were then heated at 56° C., unless otherwise stated, and stored at −20° C.

Serum samples to be tested were similarly stored at −20° C. Steer serum was stored in a CO_2 chest at −76° C. and was not thawed until immediately before use. The same lot of steer serum was employed in all experiments. It was titered for natural agglutinins against each new antigen, and the lowest dilution which did not agglutinate the spirochetes was used in the test (1 : 7 to 1 : 15 dilution).

In performing the tests, the antigen was pre-

Table 1. Effect of absorptions with VDRL antigen on the agglutinating titer of syphilitic serum

Added to tests	Agglutinating titers [1]			
	Control pool B (VDRL 1:32)		Absorbed pool B (VDRL negative)	
	10 minutes	2 hours	10 minutes	2 hours
Saline	Negative	1:40	Negative	Undiluted.
Steer serum.	1:80	1:160	Undiluted	1:40.

[1] Antigen heated at 56° C. for 40 minutes.

sensitized by the antibody before adding the steer serum, and parallel tests were run without steer serum. One-tenth cubic centimeter of antigen was mixed with 0.1 cc. of test serum or dilution in Wassermann tubes and the mixtures were shaken for either 2 or 23 hours. One-tenth centimeter of steer serum or saline was then added and the tubes were shaken for an additional 10 minutes. The tests were shaken on a standard Kahn shaker, or on a much less vigorous shaker with a rotary motion (250 revolutions per minute, 1-inch diameter). The tests were incubated either at room temperature (21° to 24° C.) or in an incubator at 33° to 35° C. Although agglutination occurred more rapidly on the Kahn shaker, the titers obtained in 2 hours on the positive control syphilitic serum pool B were similar on both shakers and were unaffected by the temperature of incubation. The method of reading the tests has been described (*4*). In the present study, titers are expressed as the lowest dilution which showed strongly positive agglutination (3+ to 4+).

Nonsyphilitic serum containing reagin antibody was produced by the method of Eagle (*7*) in normal rabbits whose serum initially showed negative VDRL (*8*) and agglutination tests. The animals were injected with saline suspensions of washed lipoidal antigen-antibody precipitate obtained by absorbing human syphilitic serum with VDRL antigen. (Twenty-five cubic centimeters of serum, VDRL titer 1:8, were absorbed once with the washed sediment from 125 cc. of antigen. Thirty cubic centimeters of serum, VDRL titer 1:64, were absorbed three times with sediment from a total of 525 cc. of antigen.) Each dose was contained in a volume of 5 cc. Five rabbits were inoculated intraperitoneally with 5 doses each during a period of 13 days and were bled on the day following the last injection.

Results

A first experiment showed the presence of at least two agglutinating antibodies in syphilitic serum. A portion of human syphilitic serum pool B, which contained both reagin and TPI antibodies, was absorbed with VDRL antigen until a negative VDRL slide test was obtained. The agglutination titers of the control serum and of the reagin-absorbed serum were then compared in a test with antigen which had been heated at 56° C. for 40 minutes. The tests were read after incubation periods of 10 minutes and 2 hours.

The results, shown in table 1, indicate that a part of the agglutinating activity of pool B was due to the presence of reagin. This is shown most clearly at 10 minutes in the test with steer serum, and at 2 hours in the test without steer serum. In the test with steer serum, control pool B (VDRL 1:32) showed an agglutination titer of 1:80 at 10 minutes whereas absorbed pool B (VDRL negative) agglutinated only when undiluted. In the 2-hour test without steer serum, the titer of pool B was 1:40, whereas absorbed pool B agglutinated only when undiluted. The presence of another agglutinating antibody, not identical with reagin, was demonstrated in absorbed pool B in the test with steer serum. The absorbed serum contained no meas-

Table 2. Effect of immunizing rabbits with VDRL antigen-antibody precipitate

Rabbit No.	VDRL titer	Agglutinating titer [1] (with steer serum)	TPI titer
4744	1:128	1:160	Negative.
4756	1:64	1:80	Do.
4755	1:64	1:40	Do.
4746	1:32	Undiluted	Do.
4754	1:32	do	Do.

[1] Antigen heated at 56° C. for 40 minutes. Tests read at 2 hours.

Table 3. Effect of incubation time on agglutinating titers of syphilitic serum and nonsyphilitic reagin serum

Added to tests	Agglutinating titers [1]					
	Control pool B (VDRL 1:32)		Absorbed pool B (VDRL negative)		Nonsyphilitic reagin serum (VDRL 1:64)	
	2 hours	23 hours	2 hours	23 hours	2 hours	23 hours
Saline	1:20	1:80	Undiluted	1:20	1:10	1:10
Steer serum	1:320	1:320	1:40	1:160	1:10	1:10

[1] Antigen heated at 56° C. for 40 minutes.

urable reagin but had an agglutination titer of 1:40 at 2 hours.

In a second experiment it was shown that *T. pallidum* was agglutinated by reagin antibody in nonsyphilitic serum. Five normal rabbits were immunized with lipoidal antigen-antibody precipitate as described under "Methods," and the serum from these animals was tested for the presence of reagin, agglutinating, and TPI antibodies. The agglutination tests were run with steer serum, using antigen heated at 56° C. for 40 minutes, and were read at 2 hours. The titers obtained in the three tests are shown in table 2. All of the serums gave positive VDRL and agglutination tests but negative TPI tests. The VDRL titers ranged from 1:32 to 1:128, and the agglutinating titers ranged from undiluted to 1:160. Three rabbits were high in both VDRL and agglutinating titers. In the two rabbits with low titers, the VDRL test appeared to be a more sensitive test for reagin than the agglutination test.

A study was next made of the effect of the length of the incubation period on the agglutinating titer. The serum samples from the five rabbits immunized with VDRL antigen-antibody precipitate were pooled and designated "nonsyphilitic reagin serum." Agglutination tests were run on this serum, which contained only reagin antibody; on pool B, which contained reagin and at least one additional antibody; and on absorbed pool B, from which the reagin antibody had been removed. The tests were run with antigen which had been heated at 56° C. for 40 minutes and were read after incubation periods of 2 and 23 hours. The results are shown in table 3.

The nonsyphilitic reagin serum (VDRL 1:64) showed no rise in titer on prolonging the incubation period from 2 to 23 hours. In the test with steer serum, pool B (VDRL 1:32) also showed no rise in titer after 2 hours. On the other hand, absorbed pool B (VDRL negative) showed a fourfold rise in titer between 2 and 23 hours. This slow rise in titer of the second antibody apparently was masked, or partially masked, in control pool B by the rapid agglutinating action of the reagin antibody. The addition of steer serum caused no rise in the titer of the nonsyphilitic reagin serum either at 2 or at 23 hours. In both the control and the absorbed pool B, the addition of steer serum caused a rise in titer at 23 as well as at 2 hours.

The effect of heat on the sensitivity and specificity of the antigen was investigated. In preparing the antigens for these studies, aliquot portions of the same spirochete suspension were used. One portion was not heated; a second portion was heated at 56° C. for 40 minutes, and a third portion was heated at 100° C. for 40 minutes.

The sensitivity of these antigens was tested in a first experiment with control pool B and with pool B which had been absorbed with VDRL antigen; and in a second experiment, with the nonsyphilitic reagin serum. The tests were read after an incubation period of 2 hours, with results which are illustrated in table 4. The titers of each of the three serums increased both with and without steer serum as the antigens were heated. Heating the antigen at 100° C. increased its sensitivity to the reagin antibody, as shown by the titers of the nonsyphilitic

Table 4. Effect of heat on agglutinability of antigen in syphilitic serum and in nonsyphilitic reagin serum

Serum tested	Added to tests	Agglutinating titers at 2 hours		
		Antigen not heated	Antigen heated, 56° C., 40 minutes	Antigen heated, 100° C., 40 minutes
Experiment 1:				
Pool B (control)	Saline	1:10	1:40	1:160
	Steer serum	1:20	1:320	1:1,280
Pool B (absorbed)	Saline	Undiluted (3+)	Undiluted (4+)	1:10
	Steer serum	Undiluted (4+)	1:80	1:320
Experiment 2:				
Nonsyphilitic reagin serum	Saline	Negative	1:20	1:40
	Steer serum	do	1:20	1:320

reagin serum, and to the second antibody as shown by the titers of the reagin absorbed syphilitic serum. Heating the antigen at 100° C. also markedly increased its sensitivity to the conglutinating action of steer serum. This is shown most clearly in the tests with the nonsyphilitic reagin serum. The addition of steer serum caused no rise in titer with the antigen heated at 56° C., but caused an eightfold rise with antigen heated at 100° C.

In testing the effect of heat on the specificity of the antigen, agglutination tests were run on serum samples from 19 individuals with negative VDRL and TPI tests (medical students and laboratory personnel). The undiluted serum from each donor was tested both with and without steer serum against antigen heated at 56° C. for 40 minutes and against antigen heated at 100° C. for 40 minutes. The tests with antigen heated at 56° C. were read after incubation periods of both 2 and 23 hours. The tests with antigen heated at 100° C. were read at 23 hours. Since approximately the same findings were obtained on each serum both with and without steer serum, the results of the two techniques have not been tabulated separately. The numbers of serums showing positive or negative agglutination with each antigen are listed in table 5.

With antigen heated at 56° C., 18 of the 19 normal serums were negative at 2 hours and 1 was weakly positive (1+). At 23 hours, 7 serums remained negative, and 12 were weakly positive. With antigen heated at 100° C., only 1 serum was negative at 23 hours. Of the 18 samples showing agglutination, 3 were weakly positive (2+) and 15 gave strongly positive reactions (3+ to 4+).

Discussion

The experiments show that the utilization of the agglutination technique as a diagnostic test for syphilis must await the preparation of more specific antigens. The test with heat-killed spirochetes measured at least two different antibodies in syphilitic serum. One antibody

Table 5. Effect of heat on agglutinability of antigen in undiluted normal human serum

Agglutination test procedure	Total serums tested [1] (TPI negative, VDRL negative)	Results of agglutination test		
		Negative	1+, 2+	3+, 4+
Antigen heated at 56° C.:				
Incubated 2 hours	19	18	1	0
Incubated 23 hours	19	7	12	0
Antigen heated at 100° C.:				
Incubated 23 hours	19	1	3	15

[1] Each serum was tested with and without steer serum, with similar results.

showed rapid agglutinating activity and was proved to be reagin. The second antibody acted more slowly and has not been identified. Its possible identity with the TPI antibody will be the subject of a later report.

The mechanism by which steer serum enhances agglutination has not been explained. With the reagin antibody, the reaction appeared to be accelerated. The reagin titer was higher with steer serum in tests read at 10 minutes, but no enhancement was obtained after an incubation period of 2 hours. On the other hand, in experiments with syphilitic serum from which reagin had been removed, steer serum enhanced agglutination after an incubation period of 23 hours. Whether this effect was due to an increased sensitivity of the test or to the participation of more than one antibody has not been determined. These problems, together with the preparation of more efficient antigens, are under continued study.

Summary

The presence of at least two agglutinating antibodies was demonstrated in syphilitic serum. One antibody agglutinated rapidly and was identified as reagin. The identity of the other, more slowly acting, antibody has not been determined. The sensitivity of the antigen increased in proportion to the temperature at which it was inactivated. Heating the antigen at 100° C. markedly decreased its specificity.

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