Demonstration of a Heat Stable Antigenic Substance in TPCF Antigen by Absorption Techniques

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IN THE PRECEDING paper it was shown that the antigenicity and serologic reactivity of TPCF antigen were substantially reduced, but not destroyed, by heating at 100° C. for 60 minutes. From certain observations it appeared that the immunological activity of the heated preparations was due to the presence of an antigenically distinct heat stable moiety rather than to the partial destruction of a single substance.

The validity of this assumption was borne out by results reported in the present paper. It will be shown that when human syphilitic serum or anti-TPCF rabbit serum was absorbed with heated TPCF antigen, this procedure removed the reactivity with heated TPCF antigen but caused no decline in titer with unheated TPCF antigen.

The heat stable substance in TPCF antigen appeared to be a hitherto undemonstrated antigen from *T. pallidum*, and limited studies were made of its significance in syphilitic infection. The antibody to this antigen arose later in the course of infection and disappeared less rapidly after treatment than the antibody to the heat labile portion of TPCF antigen.

Methods

The methods used were similar to those described in the preceding paper. TPCF antigen of approved reactivity for the TPCF test was supplied by Dr. Joseph Portnoy. TPCF tests were run by the original method of Portnoy and Magnuson (*9*). A half-volume test was sometimes used to save antigen. Tpcf-50 tests (*19*) were run in Dr. Portnoy's laboratory.

Serum pools E and G were human syphilitic serums obtained from the Public Health Service Rapid Treatment Center in Durham, N.C., and the Public Health Service Medical Center at Hot Springs, Ark., respectively. Serum 8047-60 was the pooled serum from two rabbits, each inoculated with 8 billion (8×10^9) heat-killed *T. pallidum* during a period of 5 months. The treponemes were killed by heating at 56° C. for 60 minutes. Anti-TPCF serum was the pooled serum from five rabbits each injected with 14 cc. of unheated TPCF antigen during a period of 2 months.

Serums were absorbed with TPCF antigen as described in the text. Seitz filter pads were thoroughly washed with saline and dried before use.

Early syphilitic rabbits had been infected by intradermal inoculation. Each animal was injected on the back in eight sites with a total dose of 144 million *T. pallidum*. Latent syphilitic rabbits had been infected by injecting 5–50 million treponemes into each testicle. When infections were terminated with penicillin, each animal received a total dose of 2,000, 8,000, or 32,000 units per kilogram of procaine penicillin G suspended with 2 percent (w/v) aluminum monostearate (PAM), injected in four equal daily doses. The effectiveness of therapy was demonstrated by negative node transfers from the treated rabbits.

Results

Effect of degree of heat on serologic reactivity of antigen. This subject was investigated using two antigens of approved reactivity in the 1:5 dilution and two pooled human syphilitic control serums. Aliquots of the antigens were heated in a water bath at 56°, 70°, or 100° C. for 60 minutes, or in an autoclave at 121° C. for 15 minutes under 15 pounds pressure. The heated samples of each antigen and an unheated control sample were diluted 1:5 and then tested.

As shown in table 1, the reactivity of the antigens was reduced in direct proportion to the temperature at which they were heated. However, reactivity was not completely abolished by heating at 100° C. or by autoclaving. These results are not in agreement with those of Portnoy and Magnuson (*9*), but it will be shown that different antigen lots vary in heat stability.

Variation in serologic reactivity of different

Table 1. Effect of degree of heat on serologic reactivity of TPCF antigen

Degree of heat	TPCF titers		
	Serum pool E tested with—		Serum pool G tested with antigen 80 (1:5)
	Antigen pool B (1:5)	Antigen 80 (1:5)	
Unheated control	1:160	1:160	1:320
56° C., 60 minutes	----------	1:80	1:160
70° C., 60 minutes	----------	1:40	1:80
100° C., 60 minutes	1:40	1:20	1:80
121° C., 15 minutes	1:20	1:10	1:40

antigens after heating at 100° C. for 60 minutes. Except in the experiment reported above, all studies of heated antigen were made with preparations heated at 100° C. for 60 minutes. In the experiments reported in the preceding paper eight TPCF antigens of approved reactivity in the 1:5 dilution were used. When heated at 100° C. and tested in this dilution they retained approximately 25 percent of their reactivity. Or, to express it in another manner, the ratio of unheated to heated activity shown by these antigens was approximately 4:1.

Seventy antigens have now been studied; many of these varied greatly in content of heat stable substance, as shown by the ratio of their unheated to heated reactivity. The approved reactive antigens were approved for use in the TPCF test in dilutions of 1:5–1:20. When some of these were heated and tested in the designated dilution, the heat stable substance was diluted out entirely and its presence could be demonstrated only by testing in lower dilutions or undiluted.

These characteristics of the antigens are illustrated in table 2, which shows the titers of two control serums when tested with six approved reactive preparations. The serums were titered with both the unheated antigens and with aliquots which had been heated at 100° C. for 60 minutes. Each antigen was tested in the dilution approved for use in the TPCF test. Also, three of the antigens were tested undiluted.

When the antigens were tested in their approved dilution, each control serum showed the same titer with all of the unheated antigens: pool E 1:160, and pool 8047–60 1:2,560. On the other hand, both serums showed great variation in titer with the diluted heated antigens. The titer of pool E varied from negative to 1:40, and the titer of pool 8047–60 varied from 1:40 to 1:1,280. The results obtained with serum 8047–60 showed that the ratio of unheated to heated activity in these antigens varied from 2:1 in antigen pool B to 64:1 in pool X67.

When the antigens were tested undiluted, the titers with unheated antigen were the same with both undiluted and diluted antigen except in the case of pool Q, which was the most highly

Table 2. Variation in serologic reactivity of different antigens after heating at 100° C. for 60 minutes

Antigen pool	Approved dilution	Dilution tested	TPCF titers			
			Serum pool E		Serum 8047–60	
			Unheated antigen	Heated antigen	Unheated antigen	Heated antigen
B	1:5	1:5	1:160	1:40	1:2, 560	1:1, 280
X67	1:5	1:5	1:160	Negative [1]	1:2, 560	1:40
M	1:15	1:15	1:160	1:10	1:2, 560	1:160
Z3	1:5	1:5	1:160	1:10	1:2, 560	1:160
		Undiluted	1:160	1:20	1:2, 560	1:640
Z5	1:5	1:5	1:160	1:20	1:2, 560	1:640
		Undiluted	1:160	1:40	1:2, 560	1:1, 280
Q	1:10	1:10	1:160	Negative	1:2, 560	1:80
		Undiluted	1:640	1:40	1:5, 120	1:1, 280

[1] In 1:10 dilution.

Table 3. Removal of VDRL and TPCF antibodies from serum by Seitz filtration

Serum dilution	Titers on dilutions [1] of serum 8047-60			
	VDRL		TPCF	
	Control	Filtered	Control	Filtered
1:5	1:320	1:320		
1:10	1:320	1:160		
1:20	1:320	1:160	1:1, 280	1:1, 280
1:40	1:320	1:80	1:1, 280	1:1, 280
1:80	1:320	Negative.	1:1, 280	1:1, 280
1:160	1:320	Negative.	1:1, 280	1:160
1:320	1:320	Negative.	1:1, 280	Negative.
1:640	Negative.	Negative.	1:1, 280	Negative.

[1] Filtered and unfiltered aliquots of each dilution were titered.

potent preparation. On the other hand, the titers with all three of the heated antigens were higher when they were tested undiluted. All heated antigens so far studied have shown greatest reactivity when tested undiluted.

Absorption of antibody to heat stable TPCF antigen from syphilitic serum and anti-TPCF serum. Absorption experiments were carried out with TPCF antigen of approved reactivity in the 1:5 dilution, using both unheated antigen and aliquots which had been heated at 100° C. for 60 minutes. The serum-antigen mixtures, together with unabsorbed control serums, were incubated for 3 hours in a water bath at 37° C., followed by 14 hours in a refrigerator at 5° C. The serums were tested with the same antigens which had been employed for absorption. For the TPCF tests, the unheated antigen was diluted 1:5 and the heated antigen was used undiluted.

Difficulty was encountered in separating the antigen-antibody complex from the absorbed serums. This was due to the fact that filters either failed to hold back the antigen or the antigen-antibody complex, while centrifugation only partially sedimented the antigen and tended to sediment the antibody.

In filtration experiments U F sintered glass, Selos 03 porcelain, and Seitz filters proved unsatisfactory. Of particular interest, however, was the finding, illustrated in table 3, that Seitz filters removed large amounts of both VDRL and TPCF antibody from control unabsorbed serums.

In centrifugation experiments 90 percent of the reactivity of TPCF antigen was sedimented in 4–6 hours in the ultracentrifuge at 39,000 rpm and 142,000 times G, but control serums so treated were greatly reduced in both VDRL and TPCF titers. Approximately 50 percent of the antigen reactivity was sedimented by spinning for 5–6 hours in the cold in a Servel anglehead centrifuge at 13,000 rpm and 20,000 times G. Although control serums so spun were reduced in VDRL titer, they showed no decline in TPCF titer.

The latter method of centrifugation was used to obtain antigen for absorption purposes. The sediment from 2 cc. of undiluted antigen was mixed with 2 cc. volumes of serum diluted 1:5 or 1:10. The mixtures were incubated as described above and then centrifuged in the Servel for 6 hours to sediment the antigen-antibody complex. Control serums were incubated and centrifuged along with the absorbed serums.

TPCF tests on the supernatant serums showed that one absorption only partially removed the antibody from either the human syphilitic serum or the anti-TPCF rabbit serum. Accordingly, the serums were absorbed again by the same method with the results shown in tables 4 and 5.

It is shown in table 4 that human syphilitic serum pool E was absorbed with both unheated and heated antigen. The TPCF titers of the unabsorbed control serum were 1:160 with unheated antigen and 1:40 with heated antigen. Absorbing pool E with unheated antigen com-

Table 4. Absorption of syphilitic serum with unheated and heated TPCF antigen

Syphilitic serum, human pool E (diluted 1:5)	TPCF titers	
	Unheated antigen (diluted 1:5)	Heated antigen (undiluted)
Control—unabsorbed	1:160	1:40
Absorbed with—		
Unheated antigen	Negative.[1]	Negative.
Heated antigen	1:160	Negative.

[1] In 1:5 dilution.

pletely removed the titer with both unheated and heated antigen. Absorbing with heated antigen completely removed the titer with heated antigen, but caused no decline in the titer with unheated antigen.

Table 5 shows the results of absorbing anti-TPCF rabbit serum with heated antigen. The TPCF titers of the unabsorbed control serum were 1:320 with unheated antigen and 1:80 with heated antigen. When the serum was absorbed with heated antigen, this completely removed the titer with heated antigen, but caused no decline in the titer with unheated antigen.

Significance of heat stable TPCF antigen in syphilitic infection. Serums were tested with antigens of approved reactivity in the 1:5 dilution. Unheated antigens were diluted 1:5, and heated aliquots of the same lots were used undiluted. The titers of positive serum control pool E were 1:160 with the unheated antigens and 1:40 with the heated antigens.

No positive tests on normal serums were obtained with heated TPCF antigen in testing more than 100 normal rabbit serums and 50 normal human serums. The human serums were obtained from University of North Carolina medical students and Venereal Disease Experimental Laboratory personnel.

In a group of six early syphilitic rabbits infected 1 week previously with large doses of *T. pallidum*, all had positive TPCF tests with unheated antigen with titers of 1:10–1:20. Only one showed a positive test with heated antigen with a titer of 1:10. When these animals had been infected for 9 weeks, all tests were positive. The titers with unheated antigen were 1:320–1:640 and with heated antigen were 1:80 or higher.

Table 5. Absorption of anti-TPCF rabbit serum with heated TPCF antigen

Anti-TPCF serum (diluted 1:10)	TPCF titers	
	Unheated antigen (diluted 1:5)	Heated antigen (undiluted)
Control—not absorbed	1:320	1:80
Absorbed with heated antigen	1:320	Negative.[1]

[1] In 1:10 dilution.

Eleven TPCF positive human secondary serums, which were reactive in all treponemal and nontreponemal tests, were selected from the serology evaluations and research assembly (SERA) study (*20*). Of these, eight gave positive TPCF tests with unheated antigen with titers of 1:5–1:160, and four of the eight had positive tests with heated antigen with titers of 1:5–1:40.

Table 6. Effect of curative penicillin therapy on TPCF titers of treated latent syphilitic rabbits

Rabbit No.	TPCF titers with—			
	Unheated antigen (1:5)		Heated antigen (undiluted)	
	Before treatment	5 months after treatment	Before treatment	5 months after treatment
22	1:320	1:20	1:20	1:20
28	1:640	1:80	1:80	1:80
44	1:640	1:80	1:80	1:80
45	1:320	1:40	1:40	1:40
47	1:640	1:80	1:40	1:40
48	1:160	1:20	1:40	1:40

The effect of treatment on TPCF titers was studied in latent syphilitic rabbits. Animals with infections of 12–15 months' duration were bled, and some were then given curative penicillin therapy. Five months later the treated and untreated rabbits were bled again, and conventional TPCF tests were run on the serums. The titers of the 7 control rabbits remained unchanged, but 8 of 22 treated rabbits showed a significant decline in titer at the second bleeding.

Serums from six control rabbits and from six of the treated rabbits which had declined in titer were run again in TPCF tests with both unheated and heated antigen. The initial titers of the control rabbits were 1:160–1:320 with unheated antigen and 1:20–1:40 with heated antigen, and no change had occurred in the individual titers 5 months later. The TPCF titers on the treated rabbits are shown in table 6. Before treatment the titers were 1:160–1:640 with unheated antigen and 1:20–1:80 with heated antigen. Five months after treatment the titers had fallen to 1:20–1:80 with

unheated antigen and remained 1:20–1:80 with heated antigen. (The titer with unheated antigen was not a measurement of antibody to the heat stable substance since the unheated antigen was diluted 1:5 and the heated antigen was used undiluted.)

Discussion

It was demonstrated by absorption techniques that TPCF antigens contained an antigenically distinct substance or complex which was stable to heating at 100° C. for 60 minutes. Different antigens varied greatly in their content of this substance.

In experiments not reported here the antibody to heated TPCF antigen was demonstrated in the serum of rabbits infected with yaws, *Treponema cuniculi*, or bejel, as well with syphilis, and in syphilitic serum from which reagin had been removed by absorption with VDRL antigen. Also, its presence was demonstrated by the tpcf-50 test (*19*).

Of particular interest in the present study were the findings in regard to the persistence of this antibody in treated latent syphilitic rabbits. In TPCF tests 5 months after treatment, 8 of 22 rabbits showed a significant decline in titer with unheated antigen, but no decline in titer with heated antigen. In experiments described in the preceding paper rabbits were tested 2½ years after treatment, and each of 24 animals showed the same titer with unheated and heated antigen, both diluted 1:5. In the light of the present study, it seems probable that the TPCF tests on the earlier rabbits measured only the antibody to the heat stable portion of the antigen.

Although the present studies were curtailed because of lack of antigen, it would be of the greatest interest to compare the persistence of the antibody to heat stable TPCF antigen with the persistence of the TPI antibody in both untreated and treated latent syphilis. While certain results reported in the fourth paper of this series suggest that these two antibodies may be in some degree related, there is no evidence that they are identical.

Summary

The reactivity of TPCF antigens was reduced in direct proportion to the temperature at which they were heated.

Different lots of antigen showed marked variation in reactivity after heating at 100° C. for 60 minutes.

Absorption of human syphilitic serum with unheated TPCF antigen removed the reactivity with both unheated antigen and antigen which had been heated at 100° C. for 60 minutes.

Absorption of human syphilitic serum or anti-TPCF rabbit serum with heated antigen removed the reactivity of both serums with heated antigen but caused no decline in their titers with unheated antigen.

The antibody to heated TPCF antigen arose later in syphilitic infection than the antibody to unheated TPCF antigen.

Treated latent syphilitic rabbits which showed a significant decline in titer with unheated antigen showed no decline in titer with heated antigen.

Relationship Between TPCF Antibody and Reagin

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IN EXPERIMENTS reported in the first paper of this series it was demonstrated that injections of TPCF antigen caused the development of TPCF antibodies in normal rabbits, and this was accompanied by the development of reagin in a large proportion of animals. On the other hand, our unpublished experiments and also studies of Portnoy and Magnuson (*9*) have shown that the removal of reagin from numerous syphilitic serums by absorption with VDRL antigen caused no decline in their TPCF antibody titers.

These apparently conflicting findings suggested the possibility of a cross relationship between TPCF antibody and reagin, or that under some conditions the TPCF test might