4 STUDIES on Treponema pallidum Complement Fixation Antigen

Antigenicity in Rabbits and Relation to Immunity

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IT IS WELL established that infection with *Treponema pallidum* confers resistance to reinoculation (*1*), but the mechanisms of this immunity remain obscure and a protective antigen in the organism has not been demonstrated. Attempts to vaccinate rabbits with killed treponemes have been either unsuccessful (*2–5*) or unconfirmed (*6*), and it has been shown that there is no consistent relationship between the state of immunity and the presence of reagin (*2,3,5*), TPI (*5,7,8*), or RPCF antibody (personal communication from G. R. Cannefax) in syphilitic serum.

The chemical extraction of a specific complement-fixing antigen from *T. pallidum* (TPCF antigen) by Portnoy and Magnuson (*9*) provided a new tool for further investigations of mechanisms of immunity in syphilis. The present paper reports a study of the antigenicity of this material in rabbits. It will be shown that normal rabbits injected with TPCF antigen developed TCPF antibody but were not immune to inoculation with minimal doses of *T. pallidum*.

Other findings reported here indicated that TPCF antigen might be a complex of reacting substances. This was suggested by results obtained with heated antigen and by the observation that TPCF antigen injected into rabbits

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The fourth paper, "Relationship of TPCF Antibody to Three Other Antibodies in Syphilitic Serum," was presented in part at the 11th annual symposium on Recent Advances in the Study of Venereal Diseases, at Chicago in April 1960.

References for the four papers are cited at the end of this presentation.

engendered the production of reagin in a large proportion of animals.

Methods

TPCF antigen was supplied by Dr. Joseph Portnoy. The antigens used for testing were of approved reactivity, as designated by Dr. Portnoy (personal communication). Because of the prohibitive cost of approved preparations, rabbits were injected with antigen of substandard reactivity.

Adult male rabbits of mixed breeds were employed in all experiments. The animals were housed in animal rooms in which the temperature was maintained at or below 70° F. Treponeme suspensions were prepared with the Nichols strain of *T. pallidum.*

Syphilitic infections were produced in rabbits by inoculating 5–50 million *T. pallidum* into each testis. When infections were terminated with penicillin, each rabbit received a total dose of 32,000–64,000 units per kilogram of calcium penicillin G in peanut oil containing 6 percent beeswax (POB), given in four equal daily injections (*10*).

Rabbits were inoculated intravenously with TPCF antigen in doses of 1 cc. In each experiment one group of animals received unheated antigen and a similar group received an aliquot portion of the same antigen which had been heated at 100° C. for 60 minutes.

Rabbits were inoculated intraperitoneally with suspensions of heat-killed treponemes in volumes of 2–20 cc. All treponeme suspensions were prepared, as earlier described (*5,11*), from the testicles of rabbits with early syphilitic orchitis. The organisms in each suspension were enumerated by the method of Magnuson, Eagle, and Fleishman (*12*). In preparing suspensions of living organisms used for producing infections or for challenge, 40 percent serum saline was employed both for extracting the testicles and for making subsequent dilutions. In preparing treponeme suspensions to be heated, testicles which had been frozen at −78° C. for varying periods of time were extracted with saline. Aliquot portions of each suspension were heated at 56° or 100° C. for 60 minutes and stored at −78° C. until used.

Summary of Four Papers

Treponema pallidum complement fixation (TPCF) antibody is not the protective mechanism in experimental syphilis. Rabbits injected with TPCF antigen showed the presence of this antibody for 6 months before challenge, yet developed lesions after inoculation with 100 *T. pallidum.*

TPCF antigen is a complex of reacting substances. Although largely heat labile, different lots contained varying amounts of an antigenically distinct heat stable moiety which was demonstrated by absorption techniques. The antibody to heated antigen arose later in syphilitic infection and declined less rapidly after treatment than the antibody to the heat labile portion of the antigen.

TPCF antibody is unrelated to reagin, although TPCF antigens were contaminated in varying degree with a substance which produced reagin when injected into rabbits and reacted with reagin in vitro. The failure of VDRL absorbed syphilitic serum to decline in TPCF titer was an unsatisfactory criterion for the absence of reagin reactive material in the antigen.

As shown by absorption methods, no relationship exists between TPCF antibody and Reiter protein complement fixation (RPCF) antibody. The antibody to the heat stable substance in TPCF antigen may be related in some degree to *T. pallidum* immobilization (TPI) antibody. TPCF antibody is definitely related to fluorescent treponemal antibody (FTA). The antibody to the heat labile portion of TPCF antigen appeared identical with the major part of the antibody complex measured by the FTA test.

The noninfectivity of the heated suspensions was demonstrated as earlier described (*5*) by negative node transfers from the rabbits into which they had been injected. The node transfers were made on the day before challenge. The node transfer rabbits and all challenged rabbits were observed for a period of 3 months. The specificity of lesions which developed was proved by dark-field studies.

Rabbits were bled from the ear and the serums were stored at −20° C. until tested. Quantitative TPCF (*9*), VDRL (*13*), and TPI (*14*) tests were run on the serums. In every

test one or more human syphilitic control serums (pools B, E, G, and AG–I) were also titered. When the reactivity of different experimental serums was compared, the serums were tested in the same assay.

TPCF tests were performed by the original method of Portnoy and Magnuson (*9*), using antigens of approved reactivity in a 1:5 dilution. All tests were run with both unheated antigen and an aliquot which had been heated at 100° C. for 60 minutes. Serums were diluted 1:10 and then in serial twofold dilutions. TPCF titers were expressed as the highest original dilution of serum giving complete or partial complement fixation. A three-tube or greater change in titer or a change from a negative to a positive test was considered significant.

TPI tests were run at the Venereal Disease Research Laboratory, Chamblee, Ga., and in the laboratory of George Cannefax at the Venereal Disease Experimental Laboratory, Chapel Hill, N.C. The tests were performed by the modifications of Portnoy, Harris, and Olansky (*15*) in regard to using an increased amount of complement (0.2 cc.) in the test, and an increased amount of thioglycollate in the medium. In tests run in Chapel Hill, the additional modification of using organisms from frozen testes (*16*) was employed.

Results

Effect of one booster injection of TPCF antigen on the antibody titers of treated latent syphilitic rabbits. Because of the scarcity of TPCF antigen available for study, the antigenicity of this material was first investigated by testing the booster effect of a single injection on the established antibody titers of latent syphilitic rabbits. The antigen was injected intravenously in doses of 1 cc. The rabbits were bled the day before and 2 weeks after inoculation. TPCF, VDRL, and TPI tests were run on the serums.

A booster injection given to untreated latent syphilitic rabbits with infections of 5–8 months' duration caused no change in the TPCF or VDRL titers. Treated latent syphilitic rabbits were then injected. These animals had infections of 7–24 months' duration which had been terminated by penicillin therapy 2½ years prior to the present study. Six rabbits were inoculated with TPCF antigen. As controls, six rabbits were inoculated with aliquots of the antigen which had been heated at 100° C. for 60 minutes, since it was reported (*9*) that certain lots of antigen were destroyed by this treatment. Six additional controls were inoculated with Pet milk (10 cc. each intraperitoneally) and six were similarly inoculated with saline.

The antigenicity of the TPCF antigen used in the present experiment was not completely destroyed by heating. All of the treated animals injected with antigen showed a significant rise in TPCF titer, although the rabbits which had received unheated antigen had titers approximately four times as high as those of the rabbits which had received heated antigen.

An investigation was then made of the effect of heat on the serologic reactivity of the antigen. The serums from all rabbits were tested with both unheated antigen and an aliquot which had been heated at 100° C. for 60 minutes. Before inoculation the titers in each of the four groups ranged from negative to 1:40, and similar titers were obtained with both unheated and heated antigen. The titers after the booster injection are shown in table 1. The control rabbits injected with milk or saline showed no increase in TPCF titer. However, all of the rabbits injected with antigen showed a significant rise in titer when the serums were tested with heated as well as with unheated antigen. The rabbits injected with unheated antigen showed titers of 1:80–1:640 in tests with unheated antigen and 1:40–1:60 in tests with heated antigen. The rabbits injected with heated antigen showed titers of 1:20–1:160 with unheated antigen and 1:20–1:80 with heated antigen. The

Table 1. TPCF titers of treated latent syphilitic rabbits after booster injection of TPCF antigen

Material injected	Range of TPCF titers [1]	
	Unheated antigen	Heated antigen
Unheated antigen	1:80–1:640	1:40–1:160
Heated antigen	1:20–1:160	1:20–1:80
Pet milk	Negative–1:40	Negative–1:40
Saline	Negative–1:40	Negative–1:40

[1] Before the booster injection, range in each group was negative–1:40 with both unheated and heated antigen.

positive control serum (human pool E) had a titer of 1:160 with unheated antigen and 1:40 with heated antigen.

VDRL tests on the serums from the 12 rabbits injected with TPCF antigen were negative both before and after the booster inoculations. TPI tests were performed on the six rabbits injected with unheated TPCF antigen, and three of these animals showed a one- to two-tube rise in titer which was consistent in three different TPI tests run on these serums. The control rabbits were not tested. However, it was earlier shown (*5*) that no increase in TPI titer occurred in 17 treated latent syphilitic rabbits after injection with heated or Mapharsen treated normal testicular suspension.

Development of antibodies in normal rabbits after injections of TPCF antigen. Two groups of normal rabbits were given intravenous injections of TPCF antigen, after which one group was challenged with *T. pallidum*, and animals from the other group were skin tested with TPCF antigen. In each group six rabbits were inoculated with unheated antigen and six were inoculated with aliquots which had been heated at 100° C. for 60 minutes. The rabbits were bled before inoculation and at varying intervals thereafter. TPCF, VDRL, and TPI tests were run on the serums. The samples drawn before inoculation were negative in the three tests.

The 12 animals to be challenged with *T. pallidum* were inoculated with 20 cc. each of TPCF antigen given in 20 doses during a period of 7 months. After seven doses in 2 months TPCF titers had reached their peak in tests with both unheated and heated antigen, and five rabbits had developed positive VDRL tests. TPCF titers then declined more than threefold during a 3-month rest but returned to their original level after one booster injection. They failed to rise any higher as a result of 12 additional doses of antigen during the following 2 months. TPI tests remained negative throughout the injection period. At the end of the inoculations the five surviving rabbits injected with unheated antigen had TPCF titers of 1:80–1:320 when tested with unheated antigen and 1:40–1:80 when tested with heated antigen. The six animals injected with heated antigen had titers of 1:20–1:80 when tested with either unheated or heated antigen.

Table 2. Development of reagin in normal rabbits injected with TPCF antigen

Material injected	Number rabbits injected	VDRL tests	
		Positive	Negative
Unheated antigen	10	5	5
Heated antigen	12	5	7

In the group of rabbits later to be skin tested, each animal received a total dose of 14 cc. TPCF antigen given in 14 doses during a period of 2 months. At the end of the injections all of the rabbits had positive TPCF tests with both unheated and heated antigen with titers similar to those described above. Five of the 11 surviving rabbits also developed positive VDRL tests.

Table 2 shows the development of reagin in the 22 surviving rabbits in both groups injected with TPCF antigen. Five of 10 rabbits became VDRL positive after receiving unheated antigen, and 5 of 12 became positive after receiving heated antigen. VDRL titers ranged from 1:2 to 1:8 in both sets of positive rabbits. In another experiment not described here, 11 additional normal rabbits were injected with TPCF antigen. All developed positive TPCF tests and five developed positive VDRL tests.

TPCF titers with unheated antigen on rabbits inoculated with TPCF antigen or heat-killed *T. pallidum* are shown in table 3. The titers on the skin-tested group are shown in table 4.

Development of antibodies in normal rabbits after injections of heat-killed T. pallidum. Since TPCF antigen was not destroyed by heating at 100° C., the effect of this temperature on the antigenicity of *T. pallidum* was investigated. Normal rabbits were inoculated intraperitoneally with a total individual dose of 12 billion (12×10^9) heat-killed treponemes during a period of 7 months, and were then challenged. Six rabbits were given treponeme suspension which had been heated at 100° C. for 60 minutes, and as controls six were given aliquots which had been heated at 56° C. for 60 minutes. The rabbits were bled before inoculation and at intervals thereafter. TPCF and VDRL tests were run on the serums. The preinoculation serums were negative in both tests.

All of the rabbits had positive TPCF and VDRL tests after they had received 0.7 billion treponemes in 1 month. Peak titers were obtained in both tests after the rabbits had received a total of 4.3 billion organisms in 2 months. TPCF titers with both unheated and heated antigen remained at their peak until the end of the injections 5 months later, while VDRL titers declined approximately 50 percent during this period.

At the end of the injections the four surviving rabbits which had received treponemes killed at 56° C. had TPCF titers of 1:160–1:2,560 in tests with unheated antigen, and 1:80–1:160 in tests with heated antigen. VDRL titers were 1:4–1:256. The four surviving rabbits inoculated with treponemes killed at 100° C. had TPCF titers of 1:80–1:640 with unheated antigen and 1:80–1:320 with heated antigen. VDRL titers were 1:2–1:16. TPCF titers obtained with unheated antigen on both groups of rabbits are shown in table 3.

Challenge of rabbits inoculated with TPCF antigen or heat-killed T. pallidum. The surviving rabbits which had been inoculated with 20 cc. TPCF antigen or 12 billion (12×10^9) heat-killed treponemes during a period of 7 months were bled 2 weeks after the last injection and were challenged 1 week later. The challenging dose was 100 *T. pallidum.* Three groups of control rabbits were inoculated with 100, 10, or 1 *T. pallidum.* The doses were inoculated intracutaneously in a volume of 0.2 cc. Injections were made at a single site in the center of the back just to the right of the midline. The animals were examined for a period of 3 months for the development of lesions.

The results of challenge and TPCF titers at the time of challenge are shown in table 3. The "immunized" rabbits were not resistant to infection with 100 *T. pallidum.* All developed lesions except one animal which had received treponemes killed at 56° C. and one which had received treponemes killed at 100° C. In the controls lesions developed in six of seven rabbits inoculated with 100 organisms, in seven of seven rabbits inoculated with 10 organisms, and in two of seven inoculated with 1 organism. The incubation periods in both control and "immunized" rabbits were 25–55 days. There was no correlation between TPCF titer and the development of lesions. Of the two rabbits with

Table 3. Results of challenge of rabbits inoculated with TPCF antigen or heat-killed Treponema pallidum

Material injected	Challenging dose	Total rabbits	Results of challenge		TPCF titers at challenge
			Lesion	No lesion	
TPCF antigen	100	5	5	0	1:80–1:320
TPCF antigen heated at 100° C	100	6	6	0	1:20–1:80
T. pallidum killed at 56° C	100	3	2	1	1:160–1:2, 560
T. pallidum killed at 100° C	100	4	3	1	1:80–1:640
Controls	100	7	6	1	
Controls	10	7	7	0	
Controls	1	7	2	5	

Table 4. Results of skin tests with TPCF antigen on rabbits with circulating TPCF antibody

Rabbit group	Total rabbits	Results of skin tests	TPCF titers	
			Before skin tests	After skin tests
Inoculated with TPCF antigen	5	Negative	1:80–1:640	
Early syphilitic	5	do	1:40–1:320	
Latent syphilitic, treated	5	do	1:10–1:20	1:20–1:80
Normal controls	5	do	Negative	Negative

the highest titers (1:2,560), one developed a lesion and the other failed to develop a lesion.

Skin testing of rabbits with TPCF antigen. The following groups of rabbits were skin tested: (*a*) rabbits which had been injected with a total individual dose of 14 cc. TPCF antigen during a period of 2 months; (*b*) rabbits with early syphilis of 7 weeks' duration; (*c*) rabbits with latent syphilis of 7–27 months' duration whose infections had been terminated with penicillin 3 years prior to the present experiment, and (*d*) normal control rabbits.

Skin tests were performed with TPCF antigen of approved serologic reactivity in the 1:15 dilution. Each animal was inoculated intracutaneously with 0.1 cc. of the following six dilutions: 1:3, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. The doses were injected in three sites on both sides of the back about 1 inch below the midline. The rabbits were examined at 24, 48, and 72 hours, and then on the sixth and ninth days after inoculation. They were bled immediately before and 3 weeks after the skin injections, and TPCF tests were run on the serums.

As seen in table 4, no skin reactions were observed on any of the rabbits, although all animals except the controls showed the presence of circulating TPCF antibody at the time the antigen was injected. The amount of antigen used for skin testing was insufficient to produce TPCF antibodies in normal rabbits, but the antigenicity of the preparation was demonstrated by the fact that it caused a significant rise in the TPCF titer of four of the five treated latent syphilitic rabbits.

Discussion

The results reported here show that TPCF antibody does not represent the protective mechanism in experimental syphilis. Rabbits injected with TPCF antigen or heat-killed treponemes showed the presence of this antibody for 6 months before challenge, yet developed lesions after inoculation with 100 *T. pallidum.*

It appeared from certain findings that TPCF antigen might be a complex of reacting substances. Injection of the antigen into rabbits caused the development of reagin in a large proportion of animals. The properties of heated preparations suggested the presence of an antigenically distinct component which was stable to heating at 100° C. for 60 minutes. It is of interest in this connection that Schneider (*17*) extracted a heat stable antigen from *Leptospira icterohemorrhagiae* by a method similar to that later used to extract TPCF antigen from *T. pallidum* (*9*).

The antigenic behavior of heated TPCF antigen was unlike that of either a denatured protein (*18*) or of some substance which had been partially destroyed by heating. When rabbits were injected with unheated antigen they showed relatively high TPCF titers when tested with unheated antigen, and low TPCF titers when tested with heated antigen. On the other hand, rabbits injected with heated antigen showed similarly low titers in tests with either unheated or heated antigen, and the titers remained low in spite of continued injections of heated material. Further studies on heated TPCF antigen are reported in the following paper.

Summary

One booster injection of TPCF antigen caused a significant rise in the TPCF titer of treated latent syphilitic rabbits.

Injections of TPCF antigen caused the development of TPCF antibodies in normal rabbits. This was accompanied by the development of reagin in 15 of 33 animals.

Rabbits with circulating TPCF antibodies had negative skin tests with TPCF antigen.

Rabbits which had been injected for 7 months with TPCF antigen or heat-killed treponemes and had shown peak TPCF titers for 2–5 months before challenge were not resistant to inoculation with 100 *Treponema pallidum.*

The antigenicity and serologic reactivity of TPCF antigen were substantially reduced, but not destroyed, by heating at 100° C. for 60 minutes. Both TPCF antigen and suspensions of *T. pallidum* so heated produced antibodies in rabbits which were reactive with unheated TPCF antigen. Heated antigen also reacted serologically with human syphilitic serum and with serum from rabbits injected with unheated TPCF antigen.

The possibility that TPCF antigen contains a heat stable antigenic component is discussed.