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Antigenic Structure of *Treponema pallidum*, Nichols Strain

II. Extraction of a Polysaccharide Antigen with "Strain-specific" Serological Activity

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An ultracentrifugally homogeneous heat-stable polysaccharide preparation free from serologically reactive rabbit testicular tissue antigen, including cardiolipin, was extracted from the Nichols strain of *Treponema pallidum*, and found to react by complement-fixation with homologous rabbit sera but not with human syphilitic sera. In addition, the reactive "strain-specific" component was found to be distinct from a second reactive component within the preparation related to an antigen of *T. reiteri*.

Previous investigations have shown that the virulent Nichols strain of *Treponema pallidum*, rendered noninfectious by gamma-irradiation, is capable of inducing partial protection in rabbits despite the inactivation of heat-labile antigens by the irradiation process (14, 15, 17).

These findings suggested that heat-stable, radiation-resistant, noncardiolipin antigens, presumably polysaccharide in nature, may play a role in stimulating protective antibodies in rabbits and man. The ability to demonstrate specific heat-stable antigens within the rabbit-adapted Nichols and human strains of *T. pallidum* (18), together with the possible role of polysaccharides in acquired resistance, prompted an attempt to isolate and characterize relatively pure antigens of this nature.

In this report, it will be shown that an ultracentrifugally homogeneous, heat-stable polysaccharide preparation isolated from the Nichols strain reacts, by complement-fixation, with homologous rabbit sera but not with human syphilitic sera, thereby providing evidence either for differences in host response to treponemal polysaccharide antigens or for antigenic dissimilarity among strains. Further, it will be shown that (i) the reactive "strain-specific" component is distinct from a second reactive component within the preparation which is related to an antigen of *T. reiteri* and (ii) the preparation is free from serologically reactive rabbit testicular tissue antigens, including cardiolipin.

MATERIALS AND METHODS

Polysaccharide (TP_0) extraction. The methods employed in the preparation of the treponemal suspensions, the ultrasonic disruption of the cells, and the separation of the lysate from the residue have already been described (18). The lysate was prepared from a total of 1.48×10^{10} washed cells of *T. pallidum*, Nichols strain, suspended in 54 ml of 0.14 M saline to give a final concentration of 2.75×10^8 treponemes/ml. The polysaccharide was extracted from the preparation according to the method of Raistrick and Topley (19). Briefly, 15 mg of trypsin and 0.45 ml of 1% Merthiolate were added to 45 ml of the ultrasonic lysate and incubation was carried out at 37 C for 4 days. During the second day of incubation, an additional 15 mg of trypsin was added. The *p*H was checked daily and, if necessary, adjusted to 8.5. At the end of the incubation period, the lysate was centrifuged at 19,000 × *g* for 1 hr, and 121.5 ml of absolute ethyl alcohol (68% concentration by weight) was slowly added to the supernatant. The grayish-white precipitate which formed was allowed to settle overnight in the cold, sedimented by centrifugation at 1,000 × *g* for 30 min, and then dried in vacuo over $CaCl_2$. A total of 5.51 mg of dried material was obtained from the original 45 ml of lysate. The precipitate was reconstituted in 45 ml of 0.14 M saline, heated in a water bath at 100 C for 60 min, and centrifuged at 1,000 × *g* for 30 min; no evidence of insoluble residue was detected in the preparation after the heating and centrifugation process. The antigen was stored at −20 C until use.

Antisera. Individual and pooled rabbit syphilitic sera were obtained from animals infected with the Nichols strain of *T. pallidum* 1.5 to 9 months prior to sacrifice.

Individual and pooled human syphilitic sera were obtained from patients with reactive *T. pallidum* immobilization and Reiter protein complement-fixation (RPCF) tests.

Biological false-positive (BFP) sera were obtained from five patients with no history or clinical manifestations of syphilis; Venereal Disease Research Laboratory (VDRL) and Kolmer titers of these sera ranged from 1:4 to 1:256, and they were nonreactive in RPCF and *T. pallidum* immobilization tests.

Antisera against both *T. reiteri* and its lipopolysaccharide-protein (RP) complex were prepared in rabbits as described by DeBruijn (6).

Antisera against rabbit testicular tissue homogenate were prepared in guinea pigs by the intramuscular injection of serologically nonreactive animals three times a week for 3 weeks. The dosage per injection was increased from 0.5 ml the first week to 1.5 ml the third week. The animals were given a single injection of 1.5 ml on the fourth week for a total of 10.5 ml of homogenate (0.2 g/ml) per guinea pig. The animals were rested for 1 week, and then bled via cardiac puncture. Anti-rabbit testicular tissue sera were also prepared in goats according to the method described by Julian, Portnoy, and Bossak (9).

Both animal and human sera were inactivated in a water bath for 30 min, the former at 61 C and the latter at 56 C.

Serological procedures. The serological activity of the TP_0 preparation was determined by the one-fifth volume complement-fixation (CF) technique employing 1.5 exact units of complement (1) and by the slide gel diffusion precipitin method (13). The CF test results were based upon reading after a 30-min secondary incubation period.

The RPCF test with unheated RP antigen and antigen heated in a water bath at 100 C for 60 min was carried out as described by Bekker (1).

The one-fifth volume Kolmer and VDRL tests were as described in "Serologic Tests for Syphilis" (20), except that 0.1 ml of serum was used in the former procedure.

The *T. pallidum* immobilization test was performed with the addition to the sustaining medium of 100 μg of lysozyme per ml (3, 11).

Absorption procedures. Absorption with unheated RP antigen was carried out according to the method of DeBruijn (5). By this procedure, removal of antibody to both the heat-labile protein and heat-stable lipopolysaccharide was accomplished.

The technique for cardiolipin absorption was that described by Hardy and Nell (8).

Chemical and physical determinations. The neutral polysaccharide (glucose) content of the TP_0 and rabbit testicular homogenate preparations was determined by the anthrone procedure (10), and total nitrogen was determined by the micro-Kjeldahl method.

The TP_0 preparation was analyzed for homogeneity in a Spinco model E ultracentrifuge at a speed of 59,780 rev/min for 2 hr with the use of a valve-type synthetic boundary cell.

RESULTS

Chemical and physical determinations. The polysaccharide content of the TP_0 preparation was indicated by a positive anthrone reaction of 49 μg of glucose per mg (dry weight) of antigen. Sedimentation studies in a Spinco model E centrifuge revealed a single peak, indicative of the homogeneous nature of the material.

Reactivity of the TP_0 preparation. As shown in Table 1, the TP_0 preparation was found to react with rabbit but not human syphilitic serum when 3.06 to 12.24 μg (dry weight) of antigen was used in the CF test. The reactivity with pooled rabbit syphilitic serum was confirmed by similar experiments in which individual sera from 10 rabbits infected with the Nichols strain of *T. pallidum* were used; similarly, no reactivity occurred when the TP_0 preparation was tested against individual human sera from 20 patients with unequivocal syphilis.

The identical titers observed before and after absorption of the syphilitic rabbit serum with cardiolipin indicated the absence of serologically reactive cardiolipin from the antigen (Table 2). The presence within the preparation of a noncardiolipin component specific for the Nichols strain and absent in *T. reiteri* was demonstrated by experiments in which rabbit syphilitic serum absorbed with RP antigen was used; these experiments showed that reactivity still occurred after absorption (Table 2). The slight decrease in activity of the TP_0 preparation after RP absorption suggested the presence of a second component related to a polysaccharide antigen of *T. reiteri*. This was confirmed by the CF titers of

TABLE 1. *Complement-fixation reactivity of TP_0 antigen with unabsorbed rabbit and human syphilitic sera*

Test	Pooled syphilitic serum	
	Rabbit (A)	Human (B)
TP_0 antigen (dry wt)		
12.24 μg	16[a]	NR[b]
6.12 μg	16	NR
3.06 μg	4	NR
1.53 μg	NR	NR
0.765 μg	NR	NR
Controls		
VDRL	32	2
Kolmer	64	8
RP (unheated)	128	8
RP (heated)	32	NR

[a] Reciprocal of the highest dilution of serum exhibiting 3+ to 4+ reactivity.

[b] Nonreactive with undiluted serum.

TABLE 2. *Complement-fixation reactivity of TP_0 antigen with unabsorbed, cardiolipin-absorbed, and RP-absorbed rabbit syphilitic serum (A)*

Test	Pooled rabbit syphilitic serum (A)		
	Unabsorbed	Cardiolipin-absorbed	RP-absorbed
TP_0 antigen (dry wt)			
12.24 μg	16[a]	16	8
6.12 μg	16	16	8
3.06 μg	8	8	8
1.53 μg	NR[b]	NR	NR
0.765 μg	NR	NR	NR
Controls			
VDRL	32	NR	16
Kolmer	64	NR	32
RP (unheated)	128	128	NR
RP (heated)	32	32	NR

[a] Reciprocal of the highest dilution of serum exhibiting 3+ to 4+ reactivity.

[b] Nonreactive with undiluted serum.

1:16 obtained on both anti-*T. reiteri* and anti-RP sera with undiluted TP_0 as antigen (12.24 μg, dry weight).

Precipitinogens could not be demonstrated in the TP_0 preparation by the gel diffusion method in which rabbit syphilitic, human syphilitic, anti-*T. reiteri*, and anti-RP sera were employed.

Purity relative to host tissue and antigen specificity. The identical titers obtained on syphilitic rabbit serum before and after absorption with cardiolipin provided evidence that cardiolipin antigens derived from either host testicular tissue or treponemes were absent from the preparation. This was further strengthened by the failure of the TP_0 antigen to react with undiluted sera obtained from 5 BFP reactors and 20 human syphilitics exhibiting reactive VDRL and Kolmer tests. In addition, the TP_0 antigen did not react in the CF or gel diffusion tests when guinea pig and goat antisera prepared against normal rabbit testes homogenate were used, thereby indicating the absence of reacting host tissue antigens in the preparation. Some indication of the antigen concentration which could be detected was provided by the finding that antigens present within rabbit testicular tissue in amounts of less than 0.16 μg of antigen N_2 and 0.017 μg of glucose (neutral polysaccharide) reacted with the guinea pig antitesticular tissue serum in the CF test. Similarly, the goat antitesticular tissue serum could detect 3.12 μg of antigen N_2 and 0.34 μg of glucose within the testicular tissue homogenate when the gel diffusion technique was used. The specificity of the TP_0 antigen was also indicated by its failure to react with undiluted sera from 10 randomly selected "normal" rabbits and 10 healthy human blood donors.

DISCUSSION

Attempts by other investigators to obtain tissue-free antigens from virulent *T. pallidum* have been unsuccessful (4, 12). However, in the present study, it has been possible to extract an ultracentrifugally homogeneous, heat-stable, treponemal polysaccharide (TP_0) preparation free from reacting host tissue antigens, including cardiolipin. This was accomplished by employing inactivated normal rabbit serum diluted 50% with 0.14 M saline as a suspending medium followed by differential centrifugation, washing, ultrasonic lysis, and extraction. It has been shown by CF and absorption techniques that at least two antigenic components having the same sedimentation values and reactive with rabbit but not human syphilitic sera are present within the preparation. One component is related to a polysaccharide antigen of *T. reiteri*, and the second component appears to have Nichols "strain-specific" serological activity. Their ability to react with rabbit but not human syphilitic sera parallels our previous investigations in which similar results were obtained by use of a lipopolysaccharide antigen of *T. reiteri* also found in rabbit testicular tissue (2, 7, 18). These findings, together with the demonstration that nonspecific immobilizing antigens also occur within both the Nichols strain of *T. pallidum* and rabbit testicular tissue, originally caused us to postulate that the presence of these reactive antigens might be indicative of an antigenic disparity between human and rabbit-adapted *T. pallidum*. This disparity was thought to develop as a result of a continual and intimate association between host rabbit tissue and treponemes, thereby causing the organism to assume antigens resembling those of host tissue (16). However, experiments we have recently completed have indicated that (i) the lipopolysaccharide of *T. reiteri* reacts with sera from rabbits infected with freshly isolated human strains, and (ii) the nonspecific immobilizing antigens present within both rabbit testicular tissue and the rabbit-adapted Nichols strain also occur within freshly isolated human strains after only six passages in the rabbit (*to be published*). These data lend greater support to the hypothesis that differences in host response to treponemal polysaccharide antigens account for the reactivity of TP_0 and Reiter lipopolysaccharide preparations with rabbit but not human syphilitic sera. It does not, however, rule out the possibility of antigenic dissimilarity among differ-

ent rabbit-adapted strains. Studies are being continued along these lines.

The isolation and preliminary characterization of a relatively pure polysaccharide antigen(s) from the Nichols strain of *T. pallidum* is the first step toward a more detailed elucidation of its immunogenic, physical, and chemical properties. These investigations are predicated upon previous findings which suggested that polysaccharide antigen(s) may play a dominant role in stimulating those antibodies associated with the immune response (15, 17). However, the "strain-specific" serological reactivity demonstrated in this study reemphasizes the need for exercising caution when extending to man the results of immunologic studies in the rabbit (18).

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