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Antigenic Relationships of 14 Treponemes Demonstrated by Immunofluorescence

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Immunofluorescence was used as an aid in the antigenic grouping of 14 cultivable treponemes. Antisera were prepared versus each treponemal strain, and the antiglobulins were conjugated with fluorescein isothiocyanate. A common antigen-antibody system, detected in the strains studied, was removed by absorption of each conjugate with Reiter or *Borrelia vincentii* treponemes. Thus, five categories based on shared group-specific antigens were revealed. Serogroup I: Reiter, English Reiter, Kazan, Kazan numbers 2, 4, 5, and 8. Serogroup II: Nichols and Noguchi. Serogroup III: three oral treponemes, MRB, FM, and N-39. Serogroup IV: *B. vincentii*. Serogroup V: *Treponema zuelzerae*. The five serogroups apparently are related by an immunofluorescent common antigen.

Prior studies of the treponemes, by use of agglutination, complement-fixation, and gel-diffusion methods, have revealed immunological data of taxonomic importance. Several reports gave conflicting conclusions as to the antigenic similarity (1, 7, 10) or difference (4, 11) of the Reiter and Kazan strains. Using carbolized suspensions of treponemes, Beck placed the Noguchi strain in a serological category separate from Reiter, Kroó, Kazan II, and an oral spirochete (1). Later, serological identity was established between Noguchi and the cultivable Nichols strain of *Treponema pallidum* (11). Numerous investigations have concerned the study of common and specific antigenic fractions from *T. pallidum*, Reiter (3, 5, 6, 16), *T. zuelzerae* (2), and several nonpathogenic genital treponemes (9). It has been shown that these organisms are related by a protein-type antigen. More recently, Nell and Hardy (15) extracted from the Reiter treponeme a purified polysaccharide antigen which cross-reacted with seven of nine treponemal immune sera tested. Further, a clsssification of 16 treponemes and 1 *Borrelia* species was determined by precipitating antibody methods (G. R. Cannefax, Dr. P. H. Thesis, Univ. of North Carolina, Chapel Hill, 1965).

Since it was reported (8, 14) that treponemes could be antigenically separated by direct fluorescent-antibody (FA) techniques, we have been interested in using FA techniques to confirm and extend present knowledge of treponemal classification. This report describes the antigenic relationships revealed in a study of 14 cultivable treponemes and suggests a serological grouping of the organisms.

MATERIALS AND METHODS

The treponemes included in this study were nine strains, originally purported to be *T. pallidum*, which are now nonpathogenic and cultivable on artificial media (Reiter, English Reiter, Kazan, Kazan 2, Kazan 4, Kazan 5, Kazan 8, Nichols, and Noguchi), three strains of small mouth treponemes representing normal human flora (MRB, FM, and N-39), a free-living treponeme isolated from California mud (*T. zuelzerae*), and the N-9 strain of *Borrelia vincentii*. This laboratory received the strains from several sources which were acknowledged in a paper by Hanson and Cannefax (13). Propagation of *T. zuelzerae* was in the serum-free Veldkamp medium (18). The other organisms were grown in Thioglycollate Medium (Difco) containing 10% normal rabbit serum.

Details of test procedure, preparation of antisera, and absorption techniques were described in a previous report (8). In brief, antisera were prepared in rabbits versus each of the 14 treponemes, with the use of 72-hr broth cultures for inoculum. The antisera were fractionated with half-saturated ammonium sulfate, and the antiglobulins were labeled (17) with fluorescein isothiocyanate at a dye-protein ratio of 1:40. Free fluorescein was removed by passage of the conjugated antiglobulins through a column of G-25 Sephadex (12). To determine the amount of antibody present, the conjugates were titrated on their homologous organisms. For test purposes, however, only undiluted portions of the conjugates were used. The purified conjugates were exposed to treponemal smears for 30 min in the direct FA procedure. Separate portions of the conjugates then were absorbed with either Reiter or *B. vincentii* to remove the common

treponemal antibody fraction (8) detected by immunofluorescence. The Reiter treponeme was chosen for absorption because of precedent. Absorption with *B. vincentii* was used to confirm the findings obtained with the Reiter treponeme. Those conjugates retaining heterologous reactivity were absorbed with cross-reacting antigens and retested.

Washed, intact treponemes were used in the ratio of three parts conjugate to one part packed organisms for absorption. This process was repeated until the absorbing strain no longer stained when tested.

RESULTS

Serological properties of unabsorbed conjugates. The cross-reactivity demonstrated by unabsorbed conjugates is presented in Table 1. The staining of five treponemes, namely, Nichols, Noguchi, MRB, FM, and N-39, with heterologous conjugates, was noticeably low in intensity (1+) or absent, as with the *B. vincentii* and *T. zuelzerae* labeled antiglobulins. Conjugates prepared against these five treponemes similarly demonstrated weak staining against each other, but reacted strongly (3+) with the other treponemes tested, including *B. vincentii* and *T. zuelzerae*. Strong heterologous reactivity occurred in all other combinations of staining.

Serological properties of conjugates absorbed with the Reiter treponeme. The staining that remained after absorption of the conjugates with the Reiter treponeme is summarized in Table 2. All reactivity was removed in the Reiter and English Reiter systems. The conjugates prepared for the five Kazan strains continued to react with the Kazan antigens, although the reactivity was minimal (1+). Nichols and Noguchi strains cross-reacted, as did the three mouth treponemes, MRB, FM, and N-39. *T. zuelzerae* labeled antiglobulin, in addition to staining the homologous antigen, stained *B. vincentii* to a lesser degree (1+). On the other hand, *B. vincentii* conjugate reacted only with *B. vincentii* antigen.

TABLE 1. *Staining reactions of fluorescein-labeled antiglobulins prepared against 14 treponemal strains*[a]

Organisms	Fluorescein-labeled antiglobulins						
	Reiter, English Reiter	Kazan, Kazan's 2, 4, 5, 8	Nichols	Noguchi	MRB, FM, N-39	*Treponema zuelzerae*	*Borrelia vincentii*
Reiter, English Reiter	+++	+++	+++	+++	+++	+++	+++
Kazan, Kazan's 2, 4, 5, 8	+++	+++	+++	+++	+++	+++	+++
Nichols	+	+	+++	+++	+	−	−
Noguchi	+	+	+++	+++	+	−	−
MRB, FM, N-39	+	+	+	+	+++	−	−
T. zuelzerae	+++	+++	+++	+++	+++	+++	+++
B. vincentii	+++	+++	+++	+++	+++	+++	+++

[a] Symbols: +++ = strong fluorescence (3+, 4+); + = minimal fluorescence (1+); − = no fluorescence.

TABLE 2. *Staining reactions of fluorescein-labeled antiglobulins absorbed with Reiter treponemes*[a]

Organisms	Fluorescein-labeled antiglobulins absorbed with Reiter treponemes					
	Reiter, English Reiter	Kazan, Kazan's 2, 4, 5, 8	Nichols, Noguchi	MRB, FM, N-39	*Treponema zuelzerae*	*Borrelia vincentii*
Reiter	−	−	−	−	−	−
English Reiter	−	−	−	−	−	−
Kazan	−	+	−	−	−	−
Kazan's 2, 4, 5, 8	−	+	−	−	−	−
Nichols	−	−	+++	−	−	−
Noguchi	−	−	+++	−	−	−
MRB	−	−	−	+++	−	−
FM	−	−	−	+++	−	−
N-39	−	−	−	+++	−	−
T. zuelzerae	−	−	−	−	+++	−
B. vincentii	−	−	−	−	+	+++

[a] Symbols: +++ = strong fluorescence (3+, 4+); + = minimal fluorescence (1+); − = no fluorescence.

Serological properties of conjugates absorbed with B. vincentii. The results obtained with conugates absorbed with *B. vincentii* are shown in Table 3. The two Reiter and five Kazan strains cross-reacted strongly (3+). Nichols and Noguchi and the three mouth treponemes reacted in the same manner as shown in Table 2. Only the homologous antigen was stained with the absorbed *T. zuelzerae* labeled antiglobulin, and all staining was removed from the *B. vincentii* conjugate.

Serological properties of conjugates absorbed with cross-reacting antigens. Reciprocal absorption of Reiter and English Reiter conjugates removed all staining. Absorption of the Reiter conjugate with Kazan treponemes reduced, but did not eliminate, reactivity with Reiter antigens. Absorption of each Kazan conjugate with Reiter similarly reduced reactivity with the Kazan strains. All reactivity was removed from Kazan conjugates after cross-absorption with the five Kazan strains. A summary of these results is presented in Table 4.

The labeled antiglobulins of Nichols and Noguchi did not react after absorption between these organisms. Staining differences demonstrated with the three mouth treponemes are shown in Table 5. Absorption of N-39 conjugate with either MRB or FM rendered the conjugate specific for the N-39 strain. When absorbed with N-39, the FM and MRB conjugates retained staining for both FM and MRB spirochetes. Cross-absorption of the labeled anti-FM or anti-MRB globulins completely removed reactivity for the two strains.

Although absorption of *T. zuelzerae* conjugate with *B. vincentii* removed the cross-staining indicated in Table 2, it did not seem to affect the intensity of fluorescence on the homologous anti-

TABLE 3. *Staining reactions of fluorescein-labeled antiglobulins absorbed with Borrelia vincentii*[a]

Organisms	Fluorescein-labeled antiglobulins absorbed with *B. vincentii*					
	Reiter, English Reiter	Kazan, Kazan's 2, 4, 5, 8	Nichols, Noguchi	MRB, FM, N-39	*Treponema zuelzerae*	*B. vincentii*
Reiter	+++	+++	−	−	−	−
English Reiter	+++	+++	−	−	−	−
Kazan	+++	+++	−	−	−	−
Kazan's 2, 4, 5, 8	+++	+++	−	−	−	−
Nichols	−	−	+++	−	−	−
Noguchi	−	−	+++	−	−	−
MRB	−	−	−	+++	−	−
FM	−	−	−	+++	−	−
N-39	−	−	−	+++	−	−
T. zuelzerae	−	−	−	−	+++	−
B. vincentii	−	−	−	−	−	−

[a] Symbols: +++ = strong fluorescence (3+, 4+); − = no fluorescence.

TABLE 4. *Staining reactions of two Reiter and five Kazan fluorescein-labeled antiglobulins after heterologous absorption*[a]

Fluorescein-labeled antiglobulins	Absorbed with	Organisms						
		English Reiter	Reiter	Kazan	Kazan 2	Kazan 4	Kazan 5	Kazan 8
English Reiter	Reiter	−	−	−	−	−	−	−
Reiter	English Reiter	−	−	−	−	−	−	−
	Kazan	+	+	−	−	−	−	−
Kazan	Reiter	−	−	+	+	+	+	+
	Kazan's 2, 4, 5, 8	−	−	−	−	−	−	−
Kazan 2, 4, 5, 8	Reiter	−	−	+	+	+	+	+
	Kazan	−	−	−	−	−	−	−

[a] Symbols: + = minimal fluorescence (1+); − = no fluorescence.

TABLE 5. *Staining reactions of three mouth treponeme fluorescein-labeled antiglobulins after heterologous absorption*[a]

Fluorescein-labeled antiglobulins	Absorbed with	Organisms			
		MRB	FM	N-39	All other strains
N-39	Reiter	+++	+++	+++	−
	MRB	−	−	++	−
	FM	−	−	++	−
MRB	Reiter	+++	+++	+++	−
	N-39	++	++	−	−
	FM	−	−	−	−
FM	Reiter	+++	+++	+++	−
	N-39	++	++	−	−
	MRB	−	−	−	−

[a] Symbols: +++ = strong fluorescence (3+, 4+); ++ = good fluorescence (2+); − = no fluorescence.

gen. The reactivity of *B. vincentii* conjugate was not affected by absorption with *T. zuelzerae*.

DISCUSSION

The cross-reactivity demonstrated with unabsorbed conjugates indicates the presence of a shared or common factor detected by FA in the 14 treponemal strains. Removal of the factor could be accomplished by absorption of the labeled antibodies with Reiter or *B. vincentii* spirochetes. Our observations extend the work of Deacon and Hunter (8), who demonstrated a shared antigenic fraction in four treponemes, and they support the conclusions that a common antigen is present in many cultivable and pathogenic spirochetes (2, 3, 5, 6, 9, 16). It should be noted, however, that the common antigen detected by FA may or may not be the same as that detected by complement-fixation, agglutination, or precipitin methods.

The reduced staining or lack of staining noted with Nichols, Noguchi, and the three mouth treponemes may be due to differences in structure or location of the cross-reacting antigen, such differences rendering the antigen undetectable by our methods. This theory was proposed earlier to explain lack of staining with mouth treponemes (8); however, the present study does not attempt to clarify this view.

On the basis of absorption of the labeled globulins with Reiter or *B. vincentii*, five distinct antigenic categories were demonstrated among the 14 organisms tested.

Serogroup I consists of Reiter, English Reiter, Kazan, and Kazan numbers 2, 4, 5, and 8. Cross-staining and absorption results indicate that the Reiter and English Reiter strains are an antigenic entity, as are the five Kazan strains. Although there appears to be a slight serological difference between the Reiter and Kazan strains, the difference is not considered sufficient to establish serotypes. The interpretation that a close but not identical relationship exists among the two Reiter and five Kazan strains supports the findings of Eagle and Germuth (11) and Christiansen (4). Christiansen postulated that purified polysaccharide fractions from Reiter and Kazan II were serologically identical, but that Kazan II contained at least one antigen specific for that strain. The conclusions presented here, however, conflict with the work of Beck (1), Dardanoni and Zaffiro (7), and Dupouey (10), who found Reiter and Kazan identical.

The cultivable Nichols and Noguchi strains comprise serogroup II, as demonstrated by absorptions of their labeled antibodies with Reiter or *B. vincentii*. The two organisms share an antigen that is not demonstrable in the other strains, thus justifying the placing of them in a separate serogroup. Eagle and Germuth (11) placed the Nichols and Noguchi strains in the same category because of their parallel and quantitatively equal decrease in agglutination activity upon cross-absorption.

After removal of common antibody, the three oral treponemes reveal an antigen which separates them into serogroup III. In addition, MRB and FM contain an antigen not present in N-39, whereas N-39 has an antigen not found in either MRB or FM. These findings indicate a further breakdown into serotypes. N-39 represents one serotype, whereas MRB and FM represent another.

The N-9 strain of *B. vincentii* comprises serogroup IV, and serogroup V is composed of *T. zuelzerae*. Separation of these treponemes is clearly indicated by the failure of cross-absorption to reduce homologous staining. Further, serogroups IV and V may have a minor antigen in common, as evidenced by the failure of Reiter absorption to separate completely *B. vincentii* and *T. zuelzerae* (Table 2).

Thus, the 14 treponemes are divided into five serogroups on the basis of shared group-specific antigens. The five serogroups are related to each other by a common treponemal antigen detected by FA. Limited blocking or inhibition experiments have been done which verify the serological groupings demonstrated by absorption of the conjugates with Reiter and *B. vincentii* treponemes. A summary of the antigenic groupings is given in Table 6.

TABLE 6. *Grouping of 14 treponemes based on shared group-specific antigens*

Serogroups[a]				
I	II	III	IV	V
Reiter	Nichols	MRB	*Borrelia vincentii*	*Treponema zuelzerae*
English Reiter	Noguchi	FM		
Kazan		N-39		
Kazan 2				
Kazan 4				
Kazan 5				
Kazan 8				

[a] These groups are serologically related to each other by a common treponemal antigen, as detected by immunofluorescence.

Three of the four treponemes studied previously —Reiter, *T. microdentium*, *T. zuelzerae* (8)— were individually representative of three of the five categories described here. Although limited work has been done on the fourth treponeme, *T. pallidum*, it appears to share only the immunofluorescent common antigen with the 14 nonpathogenic spirochetes.

Nell and Hardy (15) recently isolated a pure polysaccharide antigen from the Reiter treponeme which was common to Kazan A, Kazan 2, Kazan 4, PKOT, FM, and the N-9 strain of *B. vincentii*. However, this antigen was not common to Noguchi, cultivable Nichols, or the pathogenic *T. pallidum*. This polysaccharide antigen apparently is not the same as that detected by FA, for the FA common antigen was found in all the treponemes studied, including Noguchi and the cultivable and pathogenic Nichols strain of *T. pallidum*.

The classification of 16 treponemes and 1 *Borrelia* strain proposed by Cannefax (Dr.P.H. Thesis, Univ. of North Carolina, Chapel Hill, 1965), who used common and noncommon precipitin criteria, is in agreement with the serological categories reported here.

It is apparent that the treponemes can be classified efficiently according to immunological criteria. The different numbers assigned to the same serological grouping in this and other reports (9; G. R. Cannefax, Dr.P.H. Thesis, Univ. of North Carolina, Chapel Hill, 1965; Meyer and Hunter, Bacteriol. Proc., p. 79–80, 1964) point up a need for agreement on a grouping system to prevent confusion in future literature.

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