Antigen analysis of cultivable Reiter and Nichols treponemes

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During the last few decades, the study of treponemal antigens has been accomplished by several preparative techniques and detection methods. Of the cultivable treponemes, the Reiter organism has been investigated the most thoroughly. Heymann and Siefert (1962) and Nell and Hardy (1966) have presented the most complete studies of the biochemistry and antigenic properties of this organism while other investigators have studied the polysaccharide, protein, and lipid antigens. There has been little investigation of the other treponemes purported to be derived from pathogenic lesions in humans.

In syphilis, there have been limited studies of virulent *Treponema pallidum*. Portnoy and Magnuson (1955) extracted an antigen which was used in a complement-fixation test. Studies on the protein components were done by Metzger, Michalska, Podwinska, and Smogor (1969), who found a heat labile component of *T. pallidum* which was easily destroyed by physical and chemical agents. A polysaccharide isolated by Miller, Bekker, de Bruijn, and Onvlee (1969) from *T. pallidum* gave a strain specific serological result for *T. pallidum* and evidence for a common antigen with the Reiter organism.

In saprophytic treponemes, antigenic relationships have been studied by different methods. Nell and Hardy (1966) used a polysaccharide antigen to show common antigens between seven of nine treponemal strains. Cannefax (1965) used a precipitating antibody method to classify sixteen treponemes and species of *Borrelia* into five serological groups. Meyer and Hunter (1967) and Deacon and Hunter (1962), used an immunofluorescence technique to demonstrate common FA antigens between a large number of cultivable treponemes.

Since the Reiter treponeme has been so extensively studied, and the avirulent cultivable Nichols strain (Kast and Kolmer, 1933) represents another group of treponemes and is the latest treponeme to be isolated from a pathological lesion of syphilis, these two cultivable organisms were selected for the present study. The preparation, separation, characterization, and detection of the soluble antigens of the cultivable Reiter and Nichols organisms are presented.

Material and methods

CULTURE AND RUPTURE TECHNIQUES

Cultivable Reiter and Nichols treponemes were grown according to the general method of Hanson and Cannefax (1964). The organisms were washed three times with 0.1 M hydroxymethyl aminomethane (Tris)—0.14 M NaCl buffer, pH 7.8, and diluted with the same buffer to yield a concentration of 5×10^{10} organisms/ml. The optimal disruption, using the Ribi cell fractionator, was defined as being 98 per cent. of the organisms ruptured into $\frac{2}{3}$ and $\frac{1}{3}$ segments with a minimum of cell wall fragmentation as determined by darkfield and electron microscopy (Berg, Bullard, Lester, and Thornton, in preparation).

The cultivable Reiter and Nichols treponemes were disrupted in the Ribi cell fractionator at 5,000 and 9,000 lb./sq. in. respectively. The effluent material was centrifuged at $37,500 \times G$ for 30 minutes at 4°C. to separate the cellular material from the supernate (protoplast).

GEL FILTRATION

The protoplasm from Reiter organisms was fractionated by gel filtration on Sephadex G-200 (Pharmacia Fine Chemicals, Inc. Piscataway, N. J.) in 0.1 M Tris—0.14 M NaCl buffer, pH 7.8. Two upward flow columns (4.5 × 90 cm.) in tandem were used. The effluent from the columns was passed through an ultraviolet (UV) analyser and the absorption at 280 nm. was indicated on a continuous recorder (LKG Instruments, Inc., Rockville, Md.) Samples of 5 ml. were collected on an automatic fraction collector. Appropriate fractions were concentrated against

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dry carboxymethyl cellulose and stored at -90°C . until used for antigen analysis.

The protoplasm from cultivable Nichols treponemes was fractionated by gel filtration on Sephadex G-100 and followed by Sephadex G-200 using upward flow columns ($2.5 \times 90\text{ cm.}$) in tandem in 0.1 M Tris 0.14 M NaCl buffer, pH 7.8. These fractions were processed as were the Reiter treponemal fractions.

ION-EXCHANGE CHROMATOGRAPHY

Diethylaminoethyl (DEAE) cellulose Cm-32 (Whatman Laboratories, Walston Ltd. Maidstone, Kent, England) was swollen, washed, and equilibrated with 0.00875 M Sorensen's phosphate buffer solution (PBS) pH 6.3. The Reiter fraction from the first peak of the gel filtration (Fig. 1) was concentrated against dry carboxymethyl cellulose, dialysed against 0.00875 M PBS, and applied to the top of the downward flow column ($2.5 \times 35\text{ cm.}$). The eluate was monitored at 280 nm. with an UV analyser and recorder. Samples of 5 ml. were collected on an automatic fraction collector. The fractions were eluted by two buffer gradients. The first gradient was from 0.00875 M PBS, pH 6.3 to 0.4 M PBS, pH 5.2. The second gradient was from 0.4 M PBS, pH 5.2 to 0.4 M PBS—1.0 M sodium chloride (NaCl), pH 4.4. The final eluate was a buffer of 0.4 M PBS—2.0 M NaCl, pH 4.4. The Nichols fraction from the first peak of the gel-filtration was also fractionated in the same manner as the Reiter fraction. In addition, a third peak of the Reiter gel-filtration column was fractionated by the same method.

CHEMICAL DETERMINATIONS

Deoxyribonucleic acid (DNA) was determined by the method of Ceriotti (1952) modified by extraction with isoamyl acetate as suggested by Keck (1956). The standard was polymerized DNA. Ribonucleic acid (RNA) was determined by a modified orcinol reaction of Kamali and Manhoury (1969) with a yeast RNA standard. Carbohydrate content was determined by the orcinol reaction and total hexoses were determined by the anthrone reaction; both used a dextrose standard (Kabat and Mayer, 1964). Protein was determined by the method of Lowry, Rosenbrough, Farr, and Randell (1951) with a bovine serum albumin standard.

ANTIGENS

The following antigen preparations were used in the study:

- (1) *Crude antigen* This preparation was obtained directly from the Ribi cell fractionator.
- (2) *Heated crude antigen* The crude preparation was heated at 100°C . in boiling water for 60 min. and centrifuged for 30 min. at 37,500 G. and the sediment was discarded.
- (3) *Fraction A* The supernate obtained by centrifugation of the effluent from the Ribi cell fractionator.
- (4) *Heated fraction A* Fraction A was heated at 100°C . for 60 min. and centrifuged at 37,500 G., and the sediment was discarded.
- (5) *Fraction B* The insoluble cellular debris, which was obtained from the sediment left after removal of fraction

A, was washed five times with 0.1 M Tris—0.14 M NaCl buffer to remove 'most' of the soluble protein.

- (6) *Heated fraction B* Same preparative method as for the heated fraction A.

ANTISERA

These were prepared in rabbits according to the method of Deacon and Hunter (1962).

MEDIUM ANTIBODY PRODUCTION

The medium used to cultivate the organisms was centrifuged at 27,300 G. for 30 min. and then passed through a 0.22μ Millipore filter. Darkfield examinations of the treated medium were used to determine the absence of treponemes. This spent medium as well as 'normal' medium (not used for cultivation of organisms) was injected into rabbits by the intraperitoneal route (75 ml three times a week for 3 weeks) and the animals were bled one week later.

DOUBLE DIFFUSION AND IMMUNOELECTROPHORESIS

All antigens were tested against antisera. Related antisera from other treponemes (Skolidonta, Kazan, and Noguchi) were tried, as were samples of Reiter protein derived by the cryolysis method (Wallace and Harris, 1958). Double diffusion slides were prepared with 1 per cent. agarose in 0.075 M barbital buffer, pH 8.2. The reactions were allowed to develop at $4-6^{\circ}\text{C}$. with daily observations for at least 5 days. Immunoelectrophoresis was performed using the same buffer and 15 milliamps per slide for 60 min.

GROWTH PHASE ANTIGENS

The Reiter treponeme cultures were examined for appearance of antigens in different phases of the growth curve. Starting with an actively growing culture, samples were harvested and antigens prepared at the end of 24, 32, 40, 48, and 168 hours (Hanson and Cannefax, 1964).

Results

The optimal pressure used with the Ribi cell fractionator was just adequate to release protoplasmic antigens. Our studies showed that higher pressures, i.e. 1,000 to 5,000 lb/sq. in. above the optimal disruptive pressure for each organism, increased the number of fractions but decreased the reactivity of antigens, indicating a denaturing affect of the increased pressures. The profile of fractions obtained from the Reiter protoplasm with Sephadex G-200 filtration is shown in Fig. 1. The profile for the Nichols protoplasm using Sephadex G-100 filtration followed by filtration through Sephadex G-200 is shown in Fig. 2.

Another profile obtained when the effluent of the first large peak from the Reiter gel filtration was applied to a DEAE column is shown in Fig. 3. The profile from the first large peak from the Nichols gel filtration, after having been applied to a DEAE

Fraction	1	2	3	4	5
Specific Reiter antigens	+	+	+	-	-
Spent Reiter medium antigen	+	+	+	-	-
Common antigen with Nichols	+	-	-	-	-
RNA	+	+	-	-	-
DNA	+	+	-	-	-
CHO	+	-	-	-	-

RNA = Ribonucleic acid
DNA = Deoxyribonucleic acid
CHO = Carbohydrate

FIG. 1 Reiter fractionation by Sephadex G-200 filtration

Fraction	1	2	3	4	5
Specific Nichols antigens	+	+	+	-	-
Common antigen with Reiter	-	-	+	-	-
Spent Reiter medium antigen	+	-	-	-	-
RNA	+	-	-	-	-
DNA	+	-	-	-	-
CHO	+	-	-	-	-

RNA = Ribonucleic acid
DNA = Deoxyribonucleic acid
CHO = Carbohydrate

FIG. 2 Nichols fractionation. Sephadex G-100 followed by Sephadex G-200 filtration

column, is shown in Fig. 4. The effective separation of the effluent obtained from the gel filtrations is shown in Figs 3 and 4; however, we were not able to obtain these peaks by directly applying the crude material to the DEAE column.

Fraction	1	3	4	5	7	9	11
Reiter antigens	+	+	+	-	-	-	-
Spent Reiter medium antigen	-	+	+	-	-	-	-
Common antigen with Nichols	-	-	-	-	-	+	-
RNA	-	-	-	-	-	+	-
DNA	-	-	-	-	-	+	-
CHO	-	+	-	-	+	-	-

RNA = Ribonucleic acid
DNA = Deoxyribonucleic acid
CHO = Carbohydrate

FIG. 3 DEAE separation of Reiter Peak 1 from Sephadex fractionation

Fraction	1	2	3	4
Nichols antigens	-	+	+	-
Spent Nichols medium antigen	+	+	+	+
Spent Reiter medium antigen	-	+	+	-
Common antigen with Reiter	-	-	-	-

FIG. 4 DEAE separation of Nichols Peak 1 from Sephadex fractionation

WELL IDENTIFICATION Centre	CONTENT
No. 1	Rabbit (anti-Reiter A antigen) antiserum
No. 2	Reiter crude antigen
No. 3	Reiter crude antigen heated
No. 4	Reiter A antigen
No. 5	Reiter A antigen heated
No. 6	Reiter B antigen
No. 7	Reiter B antigen heated
No. 8	'Normal' medium
No. 9	Spent Reiter medium
No. 10	Spent Nichols medium
No. 10	Normal human serum

FIG. 5 *Reiter*

WELL IDENTIFICATION Centre	CONTENTS
Nos 1 and 4	Rabbit (anti-Nichols A antigen) antiserum
Nos 2 and 5	Nichols crude antigen
Nos 3 and 6	Nichols A antigen
Nos 3 and 6	Spent Nichols medium

FIG. 6 *Nichols*

Two soluble antigens in the Reiter treponeme are shown in Fig. 5, and both were found in the medium after harvest. Fig. 6 shows that the Nichols protoplasm has three soluble antigens; two like the Reiter are seen in the medium. There are probably at least three heat-stable antigens in the Reiter preparation (Fig. 7) as compared to one in the Nichols preparation (Fig. 8). There was no reaction of the control medium antigen with its antibody preparation or to the antisera of the treponemal parts or their products.

UPPER WELL	Reiter A antigen
LOWER WELL	Reiter A antigen heated
TROUGH	Rabbit (anti-Reiter A antigen heated) Antiserum

Anode on left, cathode on right

FIG. 7 *Reiter*

UPPER WELL	Nichols A antigen
LOWER WELL	Nichols A antigen heated
TROUGH	Rabbit (anti-Nichols A antigen) antiserum

Anode on left, cathode on right

FIG. 8 *Nichols*

Comparing the two strains of treponemes, a common heat-labile antigen is noted between Reiter and Nichols treponemes (Fig. 9), and also a heat-stable preparation (Fig. 10).

UPPER WELL	Nichols A antigen
LOWER WELL	Nichols A antigen heated
TROUGH	Rabbit (anti-Reiter A antigen) antiserum

Anode on left, cathode on right

FIG. 9 *Nichols*

UPPER WELL	Reiter A antigen
LOWER WELL	Reiter A antigen heated
TROUGH	Rabbit (anti-Nichols A antigen heated) antiserum

Anode on left, cathode on right

FIG. 10 *Reiter*

Results of immunodiffusion, as shown in Fig. 11, suggest that antigen production during the growth phase showed a decrease at 32 and 40 hours. At 48 hours, the lines became more dense and were similar to the 168 hour precipitin line.

WELL IDENTIFICATION Centre	CONTENTS
No. 1	Rabbit (anti-Reiter A antigen) antiserum
No. 2	Reiter antigen Lot No. 681
No. 3	Reiter A antigen—24 hr
No. 4	Reiter A antigen—32 hr
No. 5	Reiter A antigen—40 hr
No. 6	Reiter A antigen—48 hr
No. 7	Reiter A antigen—7 day

FIG. 11 *Reiter*

The Reiter antigen prepared in the Reagents sub-unit of the Venereal Disease Research Laboratory, by the cryolysis method of Wallace and Harris (1958), showed some of the same lines but these were not as dense as our preparation; however, the precipitation pattern was similar.

Immunodiffusion with Reiter protoplasm and protoplasm from Skolidonta and Kazan strains

TABLE Chemical determination of antigen preparations

Fractions	Protein ($\mu\text{g./ml.}$)	Total sugar	Hexoses	RNA	DNA
Reiter					
Crude	6,510	600	365	1,405	400
Δ Crude	450	190	60	700	335
A	2,550	450	200	1,675	420
Δ A	325	225	124	868	225
B	2,350	280	118	1,520	330
Δ B	625	105	28	315	80
Nichols					
Crude	5,250	590	210	1,210	200
Δ Crude	450	<100	125	400	168
A	3,100	190	262	1,040	230
Δ A	465	<100	197	365	148
B	1,925	583	188	390	140
Δ B	650	<100	38	150	25

Δ = heated

showed common precipitin lines. Nichols and Noguchi protoplasts showed common lines, but no common precipitin lines with avirulent treponemes were demonstrated using disrupted virulent *T. pallidum*. (Berg and others, In preparation) *T. pallidum* was harvested from rabbit testes as described by Artley and Clark (1969), and a minimum of 2×10^8 organisms was considered satisfactory for disruption. Antigens obtained from Reiter or the other cultivable treponemes listed above did not form precipitin lines with rabbit anti-*T. pallidum* antiserum.

The chemical determinations of the antigen preparations and fractions are presented in the Table.

Discussion

The disruption of treponemes for antigen analysis has been accomplished by cryolysis (D'Alessandro and Dardanoni, 1953; Wallace and Harris, 1958), ultrasonic disruption (Miller, 1969), chemical procedures (Portnoy and Magnuson, 1955) and the Ribicell fractionator (Berg and others, in preparation).

This last technique, of treponemal disruption under highly controlled conditions of pressure, provides a satisfactory method for obtaining protein antigens and facilitates the separation of axial filaments and other structures. As none of these structures has been studied extensively for antigen activity, this method adds a new dimension to treponemal antigen research.

The Reiter antigens prepared in this study are similar to those obtained by D'Alessandro and Dardanoni (1953) and Gelperin (1949). They include a protein moiety with lipid and polysaccharide. More harsh extraction of this material results in a thermostable entity similar to that of Pillot and Dupouey (1964), Christiansen (1964), and Nell and

Hardy (1966). It is a polysaccharide moiety with some RNA contamination which can be removed. This fraction has antigenic and nonantigenic components (Christiansen, 1967; Pillot and Dupouey, 1964; Berg and others, in preparation). Antigens from cultivable Nichols treponemes are similar in composition to those of Reiter treponemes. Rabbits immunized with the various Reiter and Nichols antigens showed little or no protection when challenged with virulent *T. pallidum* (Berg and others—in preparation).

The study of antigen during active growth probably indicates a priority system in the treponemes. When they are in the log phase of the growth curve, they must use all available resources for growth, for production of DNA and RNA and cell wall structure, rather than for production of antigenic material. The antigens already in the medium will continue to be seen in the precipitin reaction. It should be noted that the Reiter antigens reacted differently in terms of electrophoretic migration (Fig. 7), by staying very close to the origin instead of spreading across the slide as did the Nichols antigens (Fig. 8). This may be because the Reiter antigens were near their isoelectric point. There were also haptens noted in the parent compound, *i.e.* they were not able to produce antibody on their own but reacted with the original antigen.

Stevens (1963) fractionated Reiter protein on a Sephadex G-200 column, and this procedure was extended, by us, to derive a small amount of antigenic material from all the material present in the crude preparation. Thus the procedure could be extended to the protein antigens of virulent *T. pallidum*. If there is foreign material from rabbit tissue, it should be separated from what is virulent *T. pallidum* antigen on the DEAE column (separation by charge rather than size). The antigens that were used in this study are probably an aggregate of some kind since they were not completely independent in each peak.

This type of system, Sephadex and DEAE, can be utilized for examination of soluble *T. pallidum* antigens and should be considered when enough virulent *T. pallidum* can be cleanly prepared; however, the effort involved in preparation is formidable. In this study, to obtain an adequate amount of antigen for chemical and immunological studies, a minimum of 5×10^{10} organisms per ml. was required. Several hundred ml. of each of the Reiter and Nichols cultures were needed for this study despite the help of microtechnique modifications. Similar problems will be encountered in the harvesting of sufficient virulent *T. pallidum* for future clinical and immunological studies.

Summary

The cultivable Reiter and Nichols treponemes were disrupted in the Ribi cell fractionator at 5,000 and 9,000 pounds per square inch respectively. Six soluble antigens were separated by gel filtration and by ion-exchange chromatography from each organism. The chemical characteristics of these antigens were determined and they were also tested against specific and related rabbit anti-treponemal antisera by double diffusion and immunoelectrophoretic techniques.

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Analyse antigénique des tréponèmes cultivables de Reiter et de Nichols

SOMMAIRE

Des tréponèmes cultivables de Reiter et de Nichols furent brisés dans l'appareil de fractionnement des cellules Ribi à, respectivement, 5.000 et 9.000 livres par pouce carré. Pour chacun de ces organismes, six antigènes solubles furent séparés par filtration sur gel et par chromatographie échangeuse d'ions. Les caractères chimiques de ces antigènes furent déterminés et ils furent également testés par les techniques de double diffusion et d'immuno-électrophorèse vis-à-vis d'anti-sérums anti-tréponémiques de lapins, spécifiques ou apparentés.