Immunological studies on treponemal antigens

I. Isolation and characterization of an antigenic substance in the culture supernatant of avirulent T. pallidum (Nichols)

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Several investigators have shown that the antigens of *Treponema pallidum* are tightly bound to organisms occurring *in vivo* (Eagle and Fleischman, 1948; McLeod and Magnuson, 1953; McLeod, 1962; Christiansen, 1964). More recently Metzger, Michalska, Podwińska, and Smogór (1969) have found that the immunogenic properties of pathogenic *T. pallidum* are derived from their protein component. A serologically-active protein was isolated from a nonpathogenic strain by D'Alessandro and Dardanoni (1953). This antigen was later proved to be related to the protein fraction of the pathogenic *T. pallidum* strain (Dardanoni and Censuales, 1957; Cannefax and Garson, 1959). We considered the possibility that the protein component of cultures of avirulent *T. pallidum* may be immunogenic and provide a ready source of antigen. The present study was performed with cultured *T. pallidum* (Nichols) and the supernatant protein components were isolated and characterized for immunogenicity.

Materials and methods

PREPARATION OF SUPERNATANT

The avirulent Nichols strain of *Treponema pallidum* used for this study was obtained from Dr. G. R. Cannefax of the National Center for Disease Control, Atlanta, Georgia. Cells were grown in Spirolate broth as described by Izzat, Knox, and Wende (1971), harvested by centrifugation at 37,000 G. in a refrigerated centrifuge with a continuous flow system (Servall RC-2), and stored at -70°C. until needed. The culture supernatant was collected and sterilized by passage through a millipore filter with a pore size of 0.01 μ . The sterile supernatant was stored in containers with a 10 litre capacity at 4°C.

PROTEIN EXTRACTION WITH AMMONIUM SULPHATE

The pooled supernatant from seventeen cultures was divided into two portions containing 7,000 and 8,500 ml. respectively. As a control, 2,000 ml. cell-free medium were extracted simultaneously with the two test samples.

During the extraction procedure, all samples were kept in an ice bath. Ammonium sulphate powder was added with constant stirring to each sample until a 50 per cent. saturation had been reached. The pH of each sample was maintained between 6.8 and 7.0 with 4 per cent. sodium hydroxide. After storage overnight at 4°C., the precipitates were re-suspended in borate buffered saline, pH 7.5, then dialysed against the same buffer solution at 4°C. for 3 days with daily changes of buffer. Protein determinations, according to the method of Lowry, Rosebrough, Farr, and Randall (1951), were made on the original supernatant and cell-free medium, on the mixture obtained immediately after extraction with ammonium sulphate and on the isolated precipitate both before and after dialysis. Samples of the precipitated supernatant and cell free medium were lyophilized and used for further characterization.

GEL FILTRATION OF PRECIPITATED PROTEIN

Two 15-mg. samples of the lyophilized precipitate were re-suspended in borate buffered saline, pH 7.5, and layered on to the columns of Sephadex G-75 or G-150. Columns were eluted with either deionized water or Tris buffered saline, pH 7.2, and 5-ml. fractions were collected. The fractions were assayed for protein spectrophotometrically at 280 m μ . Fractions constituting the major peaks were pooled for further analysis. Controls consisted of the precipitated uninoculated medium and the antigen used for the FTA-ABS test (Bacto).

POLYACRYLAMIDE GEL ELECTROPHORESIS

Electrophoresis was performed by the method of Davis (1964). 25 μ g. of each sample (precipitated supernatant, precipitated medium, pooled fractions from the Sephadex columns, and FTA-ABS antigen) were subjected to a current of 5 ma for 40 min. at pH 8.4 in 7.5 per cent. acrylamide gel. Gels were stained with amido black and de-stained with 7.5 per cent. acetic acid.

TOXICITY TEST

To test the toxicity of the substances to be used for immunization, the method of Craig (1966) was followed with slight modification. Samples ranging from 5 to 12.5 mg. of the precipitated supernatant and the pooled

fractions of the precipitate from the Sephadex columns were diluted in physiological saline, and injected intradermally into three rabbits. After 18 hours, 1 ml. of 4 per cent. aqueous direct sky-blue solution was injected intravenously. Reactions were considered positive if the blue lesions were at least 5 mm. in diameter.

IMMUNIZATION OF ANIMALS

Six New Zealand white rabbits weighing between 6 and 7 lb. used for immunization were divided into two groups of three rabbits each.

In the first group, two rabbits were injected intravenously with the precipitated supernatant re-suspended in isotonic physiological saline. Injections were given every 3 to 4 days, beginning with 5 mg. and increasing by 5 mg. with each injection until a total of 270 mg. (165 mg. protein) had been given to each rabbit over a period of 5 weeks. The third rabbit was injected with the precipitated cell-free medium following the same schedule.

In the second group, two rabbits were injected subcutaneously with the precipitated supernatant suspended in complete Freund's adjuvant. Injections were given at weekly intervals for 5 weeks, according to the following schedule: 1 week 5 mg.; 2 weeks 25 mg.; 3 weeks 50 mg.; 4 weeks 100 mg.; 5 weeks 100 mg. Thus each rabbit received a total of 280 mg. (168 mg. protein) of the precipitated supernatant. The third rabbit was injected with the precipitated cell-free medium mixed with complete Freund's adjuvant following the same schedule.

All rabbits were bled weekly by puncture of the marginal ear vein. The serum was removed from the clotted blood and tested for serological activity by the VDRL, FTA-ABS, and TPI tests performed according to the Manual of United States Public Health Service (1969).

QUALITATIVE ANALYSIS OF ANTISERA

Immunodiffusion by the method of Ouchterlony and immunoelectrophoresis were performed according to the procedures cited by Weir (1967).

Results

PROTEIN STUDIES

As shown in Table I, ammonium sulphate precipitation of the culture supernatant of cells of *T.*

pallidum resulted in an increase in the protein concentration from 10 mg./ml. in the original supernatant to 62 mg./ml. in the final precipitate. In contrast, precipitation of the cell-free medium with serum resulted in 55 mg./ml. in the final concentration; and 16.50 mg./ml. when serum was omitted from the original medium composition. The precipitated supernatant as well as the two control cell-free media gave a positive reaction with the Molisch test and all were soluble in water, saline, and borate buffer.

TABLE I Solubility, protein and carbohydrate content of avirulent *T. pallidum* (Nichols strain) culture supernatant

Preparations		Molisch test	Water	Saline	Borate buffer	Protein (mg./ml.*)
Supernatant crude		+	—	—	—	10.00
Precipitate	Before dialysis	+	—	—	—	27.00
	After dialysis	+	—	—	+	26.00
	**After lyophilization	+	+	+	+	62.00
**Precipitate	With medium serum after lyophilization (control)	+	+	+	+	55.00
	Without serum after lyophilization	—	—	—	—	—
	(control)	+	+	+	+	16.50

*Modified folin ciocalten method using Beckman DK at 280 mμ (Lowry and others, 1951)

**Lyophilized precipitate diluted to contain 125 mg./ml. as a standard concentration

GEL FILTRATION STUDIES

Figs 1 and 2 show the results of gel filtration on Sephadex columns.

In the first part of this experiment, 15 mg. of either lyophilized precipitated supernatant or the same amount of lyophilized precipitated medium was layered onto a Sephadex G-75 column (30.48×2.54 cm.) and eluted with deionized water (pH 4.5). For the supernatant, a single peak was obtained with the void volume. This peak had an optical density reading of 1.9 in contrast, and the peak obtained with the control cell-free medium had an optical density reading of 0.3. This fractionation was repeated, using 24 mg. lyophilized precipitated supernatant or medium on both Sephadex G-75 and G-150 columns (45×3 cm.) eluted with deionized water (pH 4.5). The peaks obtained were similar to those shown in Fig. 1.

In the second part of the experiment, 24 mg. of the lyophilized precipitated supernatant or of the desiccated *T. pallidum* antigen used for the FTA tests were layered onto a Sephadex G-75 column (45×3 cm.) and eluted with Tris buffered saline (pH 7.2). As seen in Fig. 2, a single peak was obtained from the precipitated supernatant; this peak had an optical density reading of approximately 2.0.

FIG. 2 Column chromatography profile of ammonium sulphate extract of *T. pallidum* supernatant and Bacto FTA desiccated suspended antigen

Sephadex G-75 column 45×3 cm Eluent Tris buffer, pH 7.2
 — Supernatant — — — FTA antigen

FIG. 3 Electrophoretic patterns of *Treponema pallidum* supernatant

- (A) Lyophilized ammonium sulphate precipitated culture supernatant
 (B) Preparation A after passage through Sephadex G-75 and elution with deionized water, pH 4.5
 (C) Preparation A after passage through Sephadex G-150 and elution with Tris buffered saline, pH 7.2
 (D) Preparation A after passage through Sephadex G-150 and elution with deionized water, pH 4.5
 (E) Bacto desiccated FTA-ABS antigen

DISC ELECTROPHORESIS STUDIES

To compare the type of proteins contained in the precipitated supernatant, in the fractions obtained after gel filtration, and in the FTA antigen, disc electrophoresis was performed (see Fig. 3 on previous page). The precipitated supernatant (A) and the FTA antigen (E) gave no distinct bands because of the high concentration of protein in the two initial samples and hence the intense staining. In contrast, electrophoresis of the isolated pooled fractions obtained from gel filtration of the precipitated supernatant (B, C, D) resulted in distinguishable stained bands of protein. Fractions eluted from Sephadex columns with Tris buffered saline (C) exhibited a different electrophoretic pattern from those fractions eluted from Sephadex columns with deionized water.

TOXICITY TESTS

The precipitated supernatant as well as the pooled fractions from gel filtration of the precipitate produced lesions with a diameter of less than 5 mm. and were thus considered non-toxic.

SEROLOGICAL ACTIVITY OF RABBITS AFTER IMMUNIZATION

As shown in Table IIA and B, the sera from rabbits that had been inoculated intravenously with precipitated supernatant or lyophilized medium demonstrated no reactivity when tested by the VDRL, FTA-ABS, and TPI tests. However, the sera from rabbits inoculated subcutaneously with the same material mixed with adjuvant demonstrated reactivity to the VDRL test in the undiluted control yet

remained nonreactive with the FTA-ABS and TPI tests.

CHARACTERIZATION OF SERA FROM IMMUNIZED RABBITS Table III (opposite) shows the results after immunodiffusion of sera from both groups of rabbits with each of the following antigens: the precipitated supernatant, the FTA-ABS antigen, the precipitated medium, and the pooled fractions from both water-eluted and Tris buffered saline-eluted Sephadex columns. Antisera from rabbits inoculated intravenously with either precipitated supernatant or medium exhibited a single precipitin band with the supernatant. In contrast, sera from rabbits inoculated subcutaneously with precipitated supernatant mixed with adjuvant demonstrated two precipitin bands with the supernatant and one band with the fraction eluted from the Sephadex column with Tris buffered saline. Serum from the rabbit inoculated subcutaneously with the precipitated medium mixed with adjuvant exhibited one precipitin band with the supernatant, one band with the water-eluted fraction, one band with the FTA antigen, and one band with the homologous cell-free medium.

The sera from all rabbits were subjected to immunoelectrophoresis using the FTA-ABS antigen as the test antigen. Only the serum from the rabbit that had been inoculated subcutaneously with the precipitated supernatant mixed with adjuvant demonstrated any activity against the FTA-ABS antigen. As seen in Fig. 4 (opposite), this serum formed two precipitin bands.

TABLE II Serological changes in rabbits immunized with culture supernatant of avirulent T. pallidum

Rabbit Group	Rabbit no.	Immunization material	Initial VDRL	Serological test	Antibody reactivity after immunization
A. Intravenously immunized	1180	Supernatant without adjuvant	NR	VDRL FTA-ABS TPI	NR NR NR
	1181	Supernatant without adjuvant	NR	VDRL FTA-ABS TPI	NR NR NR
	1182	Medium + Serum without adjuvant (control)	NR	VDRL FTA-ABS TPI	NR NR NR
B. Subcutaneously immunized	1183	Supernatant with adjuvant	NR	VDRL FTA-ABS TPI	R : UND NR NR
	1184	Supernatant with adjuvant	NR	VDRL FTA-ABS TPI	R : UND NR NR
	1185	Medium + Serum with adjuvant	NR	VDRL FTA-ABS TPI	R : UND NR NR

NR = Nonreactive R : UND = Reactive undiluted

TABLE III *Precipitin response of rabbit antibody produced by the intravenous and subcutaneous administration of culture supernatant of avirulent T. pallidum*

Rabbit no.	Type of administered material	Route	Dosage (mg.)	Number of precipitin bands with isolated antigens				
				*Homologous antigen	G-75-Water fractions	G-150-Tris fractions	FTA-ABS antigen	Lyophilized medium without serum
1180 1181	Supernatant without adjuvant	Intravenous	275	1	—	—	—	—
1182 (control)	Lyophilized medium + Serum without adjuvant	Intravenous	275	1	—	—	—	—
1183 1184	Supernatant with adjuvant	Subcutaneous	280	2	—	1	—	—
1185 (control)	Lyophilized medium + Serum with adjuvant	Subcutaneous	280	1	1	—	1	1

*Identical to immunization material

FIG. 4 *Immunoelectrophoretic analysis of rabbit anti-culture supernatant serum*
WELLS: FTA-ABS Antigen
CENTRE TROUGH: Rabbit No. 1183 antiserum

Discussion

The immunological importance of the protein components present in the treponemal antigenic mosaic has been demonstrated by others (D'Alessandro and Dardanoni, 1953; Portnoy and Magnuson, 1955; Dardanoni and Censuales, 1957; Cannefax and Garson, 1959). The present report suggests the possible immunological importance of cellular related protein components in the culture supernatant of avirulent *T. pallidum*.

The culture supernatant protein components were isolated and then characterized. Precipitation of the supernatant with ammonium sulphate resulted in a higher protein concentration than that obtained in the two control cell-free media after the same precipitation. The higher concentration of protein in the culture supernatant could be due to the production and secretion by the treponemal cells of a protein substance, to the secretion by the cells of enzymes that change the composition of the original medium,

or to the debris remaining after autolysis of the treponemal cells. Although the mechanism is not understood, it is evident that the protein composition of the culture supernatant is significantly different from the protein composition of the original medium. The positive reaction of the culture supernatant with the Molisch test indicates the presence of a carbohydrate; thus the precipitated supernatant can be classified tentatively as a glycoprotein.

Gel filtration of the glycoprotein, the control medium and the FTA-ABS antigen demonstrated a different protein composition for each preparation. The fraction obtained from the supernatant has an estimated molecular weight of less than 75,000 based on the molecular sieving properties of the dextran. Electrophoresis of the fraction obtained after gel filtration of the glycoprotein demonstrated the presence of several distinct proteins.

In the Ouchterlony gel diffusion, the precipitated supernatant produced two precipitin bands when

placed against homologous antisera. Possibly one band was the result of a reaction between the glycoprotein and its specific antibody and the other band was the result of a reaction between precipitated protein in the cell-free medium and its specific antibody. Data from gel diffusion studies on the fractions obtained after gel filtration support this hypothesis. Only a single precipitin band developed when pooled Sephadex fractions from the precipitated supernatant were allowed to react with homologous antiserum. In contrast, no bands developed when antiserum to control medium was allowed to react against the same pooled fractions.

When the precipitated supernatant was injected into rabbits intravenously without adjuvant, no serological activity was demonstrable by the VDRL, TPI, or FTA-ABS tests. This absence of serological activity indicates a lack of homology between the antibody to culture supernatant and the antibodies measured by the VDRL, TPI, and FTA-ABS tests. In contrast, when the precipitated supernatant was injected subcutaneously with adjuvant, antibody was produced that reacted in the VDRL test. However, since the VDRL test was reactive also on the rabbits immunized with control medium plus adjuvant, it can be assumed that the reactivity was not due to the treponemal related protein in the culture supernatant.

A serological and immunological relationship between the culture supernatant and the FTA-ABS antigen was observed after electrophoresis. Apparently a common antigenic group exists between the two antigens as shown by the ability of the culture supernatant to induce in rabbits antibody that precipitates with the FTA-ABS antigen. In contrast, antiserum against the medium did not exhibit this phenomenon.

The above data suggest the existence of an antigenic substance in the culture supernatant of avirulent cells of *Treponema pallidum*. This antigenic substance is related in some way to the antigen used for the FTA-ABS tests.

Summary

On the assumption that some *T. pallidum* antigens are soluble rather than cell-bound, the culture supernatant of avirulent *T. pallidum* was pooled and characterized for its antigenic activity. Precipitation with 50 per cent. ammonium sulphate resulted in a complex preparation composed primarily of glycoprotein. The preparation was non-toxic to rabbits. Immunization of rabbits with the glycoprotein substance caused production of a precipitating antibody that reacted both with the glycoprotein and with the FTA-ABS antigen. Short-term subcut-

aneous immunization stimulated cardiolipin-reagin reactivity.

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Études immunologiques sur les antigènes tréponémiques

I. Isolement et caractérisation d'une substance antigénique dans le surnageat de culture de *T. pallidum* (Nichols) non virulent

SOMMAIRE

Dans l'hypothèse que certains antigènes du *T. pallidum* sont solubles plutôt que liés à des cellules, on prépara un pool de surnageats de culture de *T. pallidum* non virulent dont l'activité antigénique fut établie. Après précipitation par une solution à 50 pour cent de sulfate d'ammonium, on obtint une préparation composée principalement de glycoprotéines. La préparation ne fut pas toxique pour le lapin. L'injection de cette substance glycoprotéique aux lapins entraîna la production d'un anticorps précipitant, réagissant à la fois avec la glycoprotéine et avec l'antigène FTA-ABS. Des injections sous cutanées répétées à courts intervalles entraîna une positivité vis-à-vis de la réagine cardiolipidique.