Immunological studies on treponemal antigens

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

N. N. IZZAT, E. B. SMITH, S. W. JACKSON, AND J. M. KNOX
From the Department of Dermatology and Syphilology, Baylor College of Medicine, Houston, Texas 77025, and the Syphilology Research Laboratory, Veterans Administration Hospital, Houston, Texas 77031, U.S.A.

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, Podwinska, and Smogor (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 Dar-danoni (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 mμ. Fractions constituting the major peaks were pooled for further analysis. Controls consisted of the precipitated un inoculated medium and the antigen used for the FTA-ABS test (Bacto).

POLYACRYLAMIDE GEL ELECTROPHORESIS
Electrophoresis was performed by the method of Davis (1964). 25 μg. 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

Received for publication March 30, 1971
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.

**FIG. 1 Column chromatography profile of ammonium sulphate extract of T. pallidum supernatant**

*Sephadex G-75 column 30-48 × 2.54 cm*
*Eluent Deionized water, pH 4-5*

- **Supernatant**
- **Control medium**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Molisch test</th>
<th>Water</th>
<th>Saline</th>
<th>Borate buffer</th>
<th>Protein (mg./ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant crude</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>10-00</td>
</tr>
<tr>
<td>Precipitate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before dialysis</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>27-00</td>
</tr>
<tr>
<td>After dialysis</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>26-00</td>
</tr>
<tr>
<td><strong>After lyophilization</strong></td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>62-00</td>
</tr>
<tr>
<td><strong>Precipitate</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>With medium serum after lyophilization (control)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>55-00</td>
</tr>
<tr>
<td>Without serum after lyophilization (control)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>16-50</td>
</tr>
</tbody>
</table>

*Modified folin ciocalten method using Beckman DK at 280 mu (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.
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 II A 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

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

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

<table>
<thead>
<tr>
<th>Rabbit no.</th>
<th>Type of administered material</th>
<th>Route</th>
<th>Dosage (mg.)</th>
<th>Number of precipitin bands with isolated antigens</th>
</tr>
</thead>
<tbody>
<tr>
<td>1180</td>
<td>Supernatant without adjuvant</td>
<td>Intravenous</td>
<td>275</td>
<td>1 Homologous antigen</td>
</tr>
<tr>
<td>1181</td>
<td>Lyophilized medium + Serum without adjuvant</td>
<td>Intravenous</td>
<td>275</td>
<td>G-75-Water fractions</td>
</tr>
<tr>
<td>1182 (control)</td>
<td>Lyophilized medium + Serum without adjuvant</td>
<td>Intravenous</td>
<td>275</td>
<td>G-150-Tris fractions</td>
</tr>
<tr>
<td>1183</td>
<td>Supernatant with adjuvant</td>
<td>Subcutaneous</td>
<td>280</td>
<td>FTA-ABS antigen</td>
</tr>
<tr>
<td>1184</td>
<td>Lyophilized medium + Serum with adjuvant</td>
<td>Subcutaneous</td>
<td>280</td>
<td>Lyophilized medium without serum</td>
</tr>
</tbody>
</table>

*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'Alesandro 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 subcutaneous immunization stimulated cardiolipin-reagin reactivity.

We wish to thank Miss C. R. Wills and Miss S. E. McCotter for able technical assistance. The investigation was supported by the John A. Hartford Foundation Grant 3833 and WHO Grant V3-181-30.

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Études immunologiques sur les antigènes tréponémiques
I. Isolement et caractérisation d'une substance antigénique dans le surnageat de culture de Treponema pallidum (Nichols) non virulent

 SOMMAIRE

Dans l'hypothèse que certains antigènes du Treponema pallidum sont solubles plutôt que liés à des cellules, on prépare un pool de surnageats de culture de Treponema pallidum non virulent dont l'activité antigénique fut établie. Après précipitation par une solution à 50 pour cent de sulfate d'ammonium, on obtient 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 entraînent une positivité vis-à-vis de la réagine cardiolipidique.