Purification of TR-b, a Reiter Treponeme Protein Antigen Precipitating with Antibodies in Human Syphilitic Sera

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TR-b is a Reiter treponeme antigen, cross-reacting with an antigen in Treponema pallidum (Nichols pathogenic strain). Sera from patients with secondary syphilis contain precipitating antibodies against TR-b. The isolation of TR-b from a bacterial sonic extract is described here. It involved five fractionation steps: anion-exchange chromatography (DE-52 Whatman), gel filtration (Ac-A-22 Ultrogel), and affinity chromatography on phenyl-Sepharose CL 4B, iminodiacetic acid-Sepharose 4B, and lysine-Sepharose 4B, respectively. The purified TR-b was enriched 199 times compared with the starting material, and the recovery was 12%. TR-b was shown to be a protein; it did not bind to a series of lectins, and by gel filtration and polyacrylamide gel electrophoresis, the molecular weight was determined to be 610,000 to 630,000. It was found by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be composed of identical 70,000-dalton subunits. The isolated TR-b was immunologically pure when tested in crossed immunoelectrophoresis against polyspecific anti-Reiter immunoglobulin. The purified TR-b antigen was used for the production of a monospecific rabbit antiserum, giving strong fluorescence with both the Reiter treponeme and T. pallidum in an indirect immunofluorescence test.

Patients with secondary syphilis have precipitating antibodies against 5 of 40 antigens in the apathogenic Reiter treponeme (14). One of these five antigens (TR-a) is represented by a weak and not well-reproduced precipitate in crossed immunoelectrophoresis (IE) (14), but the remaining antigens (TR-b, TR-c, TR-d1, TR-d2, TR-e) have recently been shown to be related to corresponding antigens in Treponema pallidum (15). It is, therefore, probable that antibodies against TR-b, TR-c, TR-d1, TR-d2, and TR-e, when present in syphilitic sera, are raised in response to the corresponding antigens of T. pallidum (15). TR-e has been isolated and identified as the periplasmic flagellum or axial filament of the Reiter treponeme (11, 17). TR-d1 has been found to consist of RNA (12), and recently TR-c and TR-d2 have been purified and shown to be protein antigens (9; manuscript in preparation). TR-e has been shown to be very useful in enzyme-linked immunosorbent assays for the detection of immunoglobulin G (IgG) and IgM antibodies in syphilis (16, 18).

In this study, a method for the purification of TR-b from a sonically treated supernatant of the Reiter treponeme is presented.

MATERIALS AND METHODS

Reiter treponeme. The same strain of the Reiter treponeme as used previously (14) was grown and harvested as described elsewhere (14). Eight grams (wet weight) of treponemes was suspended in 80 ml of 0.05 M Tris-hydrochloride buffer, pH 8.0. This suspension was sonicated in an ice bath with an MSE 150-W ultrasonic disintegrator (Manor Royal, Crawley, England) with output frequency of 20 kHz and an amplitude of 20 microns for 6 × 30 s at 30-s intervals. The sonic extract was centrifuged at 35,000 × g for 15 min at 4°C, and the supernatant was used as the crude product for the purification of antigen TR-b.

T. pallidum. T. pallidum antigen for electrophoresis was prepared as described earlier (15). Rabbit antibodies. Antibodies against the Reiter treponeme were obtained by the immunization of rabbits with a crude bacterial sonic extract as previously described (14). A purified immunoglobulin fraction with a protein concentration of 15.8 g/liter was prepared from a pool of these antisera by the method of Harboe and Ingild (4). Monospecific antibodies against TR-b were obtained by the immunization of two rabbits with the purified TR-b in Freund incomplete adjuvant by the procedure described by Harboe and Ingild (4), using 10 μg of TR-b protein per injection. Rabbit anti-T. pallidum immunoglobulin was the same as described earlier (15). Human sera. Sera were obtained from selected patients with secondary syphilis with strong reactivities in traditional syphilis serological tests and from healthy persons as described earlier (14). Fused rocket IE. The fractionations were monitored by means of fused rocket IE (19).

Crossed IE with intermediate gel. Crossed IE with intermediate gel was performed as described by Axel sen (1).
Tandem crossed IE. The identity between the purified TR-b and the TR-b in the crude Reiter sonicate extract was tested by tandem crossed IE (6).

Agarose gel electrophoresis. Agarose gel electrophoresis was performed as described by Johansson (5).

Staining methods. Immunoprecipitates of TR-b were stained for RNA and DNA with "Stains All" (1-ethyl-2-[3-(1-ethyl-naphtho[1,2d]thiazolium-2-yldiene)-2-methylpropenyl] naphtho[1,2d]thiazolium bromide; Serva Feinbiochemica, Heidelberg, Germany) by the method of Gould and Matthews (3). Periodic acid-Schiff reagent (24) and Sudan black B (21) were also used for the staining of carbohydrates and lipids in the immunoplates.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a homogeneous gel with 10% acrylamide in 0.1 M phosphate buffer (pH 7.0) and 0.2% SDS by the method of Weber and Osborn (21). The protein markers were thymus tissue proteins (molecular weight, 33 x 10^3), ferritin (molecular weight, 220 x 10^3 and 18 x 10^3), albumin (molecular weight, 67 x 10^3), catalase (molecular weight, 60 x 10^3), lactate dehydrogenase (molecular weight, 36 x 10^3), phosphorylase b (molecular weight, 94 x 10^3), ovalbumin (molecular weight, 43 x 10^3), carbonic anhydrase (molecular weight, 30 x 10^3), trypsin inhibitor (molecular weight, 20.1 x 10^3), and α-lactalbumin (molecular weight, 14.4 x 10^3), all from Pharmacia Fine Chemicals, Uppsala, Sweden.

Continuous gradient PAGE. Electrophoresis in gels containing 2 to 16% and 4 to 30% continuous gradients of polyacrylamide (Pharmacia Fine Chemicals) was used to determine the molecular weight of the native form of TR-b. The electrophoresis buffer contained 0.09 M Tris, 0.08 M boric acid, and 0.003 M disodium EDTA. A kit of molecular weight proteins from Pharmacia Fine Chemicals (see above) was used for the calibration of the linear best-fit relationship between K_v and the log of the molecular weight.

Gel filtration on Ac-A-22 Ultrogel. The molecular weight of TR-b was also estimated by gel filtration on Ac-A-22 Ultrogel. The total gel bed volume (V_b) was 128 ml, and it was packed in a column with bed dimensions of 1.6 x 70 cm (K 16/70 from Pharmacia Fine Chemicals). The column void volume (V_v) was the elution volume for dextran blue 2000. The elution volume (V_e) was measured, and K_v = V_v - V_e/V_b - V_v was calculated for each of the proteins. The calibration curve was calculated for the relation between K_v and the log of the molecular weight of the known proteins. Molecular weight standards were thyroglobulin (molecular weight, 669,000), ferritin (molecular weight, 440,000), aldolase (molecular weight, 158,000), albumin (molecular weight, 67,000), ovalbumin (molecular weight, 43,000), chymotrypsinogen (molecular weight, 25,000), and RNase (molecular weight, 13,200), all from Pharmacia Fine Chemicals.

Chemical analysis. Protein concentration was determined by the method of Lowry et al. (7), with bovine serum albumin as the standard. Hexoses and pentoses were estimated by the anthrone (13) and orcinol (8) methods, respectively.

Quantification of TR-b. The quantification of TR-b was performed by rocket IE (22), using the monospecific antiserum against the purified TR-b.

Lectin binding. The binding of purified TR-b to lectins was tested in small columns and by crossed immuno-affino-electrophoresis (2). The following lectins were used: concanavalin A-Sepharose, lentil-lectin Sepharose 4B, wheat germ lectin Sepharose 6 MB, Helix pomatia lectin Sepharose 6 MB (all from Pharmacia Fine Chemicals), peanut agglutinin (IBF Réactifs, Chichy, France), and Ricinus agglutinin (Miles Laboratories, Elkhart, Ind.).

Immunofluorescence experiments. T. pallidum (Nichols pathogenic strain) and the Reiter treponeme were fixed to glass slides (20), and monospecific rabbit anti-TR-b was added to the slides. In some of the experiments, monospecific rabbit anti-TR-b was preabsorbed with various concentrations of purified flagella (11). After being washed, a fluorescein isothiocyanate-conjugated swine anti-rabbit immunoglobulin (code F 2190, lot no. 069 B, DAKOPATTS, Copenhagen, Denmark) was added to the slides, and the treponemes were microscopically tested for fluorescence. Pre-immunization serum was used as a control and gave no fluorescence.

Materials. The following gel materials were used in the isolation procedure: DEAE-cellulose (DE-52, Whatman), Ac-A-22 Ultrogel (Reactifs IBF), phenyl-Sepharose CL 4B, lysine Sepharose 4B (Pharmacia), and iminodiacetic acid Sepharose CL-4B (Pierce Chemical Co., Rockford, Ill.). The concentration step was performed on an Amicon stirred ultrafiltration cell model no. 202 with a PM 10 filter (all from Amicon Corp., Lexington, Mass.).

RESULTS

Purification procedure. An 80-ml amount of the Reiter sonically treated supernatant was added to a column of DEAE-cellulose with a bed volume of 180 ml, and elution was performed with a stepwise salt gradient, including 180 ml of each of 0.05, 0.1, 0.15, 0.25, 0.5, and 0.75 M NaCl in 0.05 M Tris-hydrochloride, pH 8.0. The flow rate was 100 ml/h. A total of 115 fractions in volumes of 10 ml each were collected, and every second fraction was tested in fused rocket IE against rabbit anti-Treponema Reiter immunoglobulin. Fractions nos. 78 to 102 containing TR-b (Fig. 1A) were pooled and concentrated 20 times. This suspension was then gel filtered at 4°C on a column (2.5 by 100 cm) with Ac-A-22 Ultrogel (Pharmacia K 25/100) with a bed volume of 480 ml. The column was calibrated with dextran blue 2,000 (Pharmacia), and the column constant (K_v) was estimated to be 52. The column was eluted with 0.05 M Tris-hydrochloride (pH 8.0) containing 0.2 M NaCl and 15 mM NaN_3 at a flow rate of 10 ml/h. A total of 100 fractions of 5 ml each were collected. The fractions were tested by fused rocket IE. TR-b was present in fractions nos. 50 to 62 (Fig. 1B), which were pooled and applied to a column of phenyl-Sepharose CL-4B with a bed volume of 8 ml. The elution of the bound antigens was obtained by a stepwise ethylene glycol gradient, using 8 ml each of 10, 20, 50, and 75% ethylene glycol in 0.05 M Tris-hydrochloride, pH 8.0. Twenty-six fractions of 3 ml each were collect-
ed, with a flow rate of 20 ml/h. TR-b was eluted in fractions nos. 2 to 6 with 10% ethylene glycol, as visualized in fused rocket IE (Fig. 1C). The pooled fractions 2 to 6 were dialyzed for 24 h against 1 liter of 0.05 M Tris-hydrochloride to remove the ethylene glycol and, thereafter, added to a column of iminodiacetic acid Sepharose CL-4B with a bed volume of 8 ml. The gel was first washed with 30 ml of 0.05 M Tris-hydrochloride to remove unbound antigens followed by specific elution with 20 ml of 10 mM EDTA in 0.05 M Tris-hydrochloride. A total of 25 fractions of 25 ml each were collected. The flow rate was 10 ml/h. TR-b was eluted with EDTA and detected in fractions nos. 20 to 25 (Fig. 1D), whereas several other contaminating treponemal antigens passed unbound through the matrix. The final purification of TR-b was obtained by affinity chromatography on lysine-Sepharose 4B. The pooled fractions nos. 20 to 25 from the iminodiacetic acid Sepharose column was added to a column of 8 ml of lysine-Sepharose 4B gel, and elution was performed with a stepwise salt gradient with 8 ml each of 0.05, 0.1, 0.25, and 0.5 M NaCl in 0.05 M Tris-hydrochloride, pH 8.0; 20 fractions of 4 ml each were collected. TR-b was eluted with 0.05 M NaCl. Fractions nos. 3 to 6 containing TR-b free of contaminating antigens were pooled, and this was the end product of the purification procedure. The yield of pure TR-b in one procedure was 2 mg of protein. Recoveries and enrichments of TR-b after the various fractionation steps are given in Table 1. The purification of TR-b was performed five times with nearly identical results.

Several results indicated that the purified TR-b is a protein. It gave an absorption maximum at 280 nm upon spectral analysis, and it reacted with the Folin reagent of the Lowry reaction. Staining for polysaccharide, lipid, RNA, and DNA was negative, and the binding experiments with six lectins all gave negative results. No detectable hexoses and pentoses were estimated by the anthrone and orcinol methods, respectively.

The purified antigen TR-b showed a reaction of identity in tandem crossed IE with the TR-b antigen in the reference pattern. Only one precipitate was seen when the isolated antigen was tested against a polyspecific anti-Reiter immunoglobulin (a-TR) in the upper gels. Arrows point to the TR-b precipitate. The fraction numbers are indicated. The plates were stained with Coomassie brilliant blue R-250.

FIG. 1. Fused rocket IE profiles of the Reiter treponemal antigens eluted from the anion-exchange chromatographic gel (DE-52 Whatman) (A), Ac-A-22 Ultrogel (B), phenyl-Sepharose CL-4B (C), iminodiacetic acid-Sepharose CL-4B (D), and lysine-Sepharose 4B (E). Samples (10 μl) from the fractions were analyzed against polyspecific rabbit anti-Reiter immunoglobulin (a-TR) in the upper gels. Arrows point to the TR-b precipitate. The fraction numbers are indicated. The plates were stained with Coomassie brilliant blue R-250.

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TABLE 1. Recovery and enrichment of TR-b in the different purification steps

<table>
<thead>
<tr>
<th>Purification step</th>
<th>% Recovery of TR-b in percentage of TR-b in Treponema Reiter sonic extract&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Enrichment of TR-b when compared with Treponema Reiter sonic extract&lt;sup&gt;b&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>Treponema Reiter sonic extract</td>
<td>100</td>
<td>1.0</td>
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<tr>
<td>DE-52 Whatman eluate</td>
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<td>13</td>
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<tr>
<td>Ac-A-22 Ultrogel eluate</td>
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<td>Phenyl-Sepharose CL-4B eluate</td>
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<td>Iminodiacetic acid Sepharose eluate</td>
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</tr>
<tr>
<td>Lysine-Sepharose 4B eluate</td>
<td>12</td>
<td>199</td>
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<sup>a</sup> Recovery was estimated by rocket IE.
<sup>b</sup> Enrichments were calculated relative to total protein, setting the amount of TR-b per gram of protein in Treponema Reiter sonically treated supernatant to 1.0.

TR-b resulted in the production of monospecific antisera. When Reiter sonic extract was submitted to crossed IE, employing the specific antiserum in the second-dimensional gel, only TR-b was precipitated (Fig. 4B). However, the precipitate representing the flagellum disappeared when the monospecific anti-TR-b was incorporated in the intermediate gel (Fig. 4B). Monospecific rabbit anti-TR-b showed strong fluorescence with both the Reiter treponema and T. pallidum in the indirect immunofluorescence assay. Strong fluorescence was also seen when the monospecific rabbit anti-TR-b was preabsorbed with purified Reiter flagella. Rabbit anti-T. pallidum immunoglobulin contained weak antibodies against the purified TR-b antigen when analyzed in crossed IE. No precipitation lines were observed when T. pallidum antigens were analyzed against monospecific rabbit anti-TR-b immunoglobulin in crossed IE.

DISCUSSION

Many commercially available affinity chromatography gels were tested in a pilot study for their capability of separating the treponemal antigens in experiments using small columns. The three affinity gels used in this study were selected due to their good separating properties. By the combination of anion-exchange chromatography and gel filtration, together with the described affinity chromatographic steps, TR-b was isolated and purified efficiently. The method described is technically easy to perform. One isolation procedure took 1 week, and all of the gel material could be reused. The method is therefore cheap for the production of TR-b on a larger scale. The reproducibility was good, i.e., five separate experiments gave similar results.

The isolated TR-b antigen was immunologically pure when tested in crossed IE against polyspecific anti-Reiter immunoglobulin, detecting more than 40 treponemal antigens. The purity of TR-b was confirmed by the finding that only one band with a molecular weight of 70,000 was produced.

FIG. 2. Crossed IE of purified TR-b antigen against polyspecific rabbit anti-Reiter immunoglobulin (a-TR) in the upper gels. The intermediate gels contained serum from healthy persons (Ø) (A) and human syphilitic serum (SS-serum) (B). The figure shows that TR-b is immunologically pure (A) and that human syphilitic serum reacts with the purified antigen (B).

FIG. 3. SDS-PAGE of isolated TR-b showing one band with an apparent molecular weight of 70,000.
by SDS-gel electrophoresis. In addition, only one band was detected in agarose gel electrophoresis.

The molecular weight of TR-b was found to be in the range of 610,000 to 630,000, which is similar to that of the previously purified TR-c antigen (9). Also, the chromatographic columns used in the purification of TR-b were the same as those used for the purification of TR-c. TR-b and TR-c were eluted together in the anion-exchange chromatography and in the gel filtration step, but different elution patterns were found for TR-b and TR-c in the hydrophobic interaction chromatography and in the two affinity chromatographic gels, iminodiacetic acid Sepharose CL-4B and lysine-Sepharose 4B. Thus, TR-b was eluted before TR-c on the phenyl-Sepharose and lysine-Sepharose columns but later than TR-c on the iminodiacetic acid Sepharose column.

The immunofluorescence experiments showed that antibodies against TR-b gave strong fluorescence with both the Reiter treponeme and T. pallidum, indicating the existence of a TR-b homologous antigen in T. pallidum. We cannot explain why TR-e (the flagellum) was absent from the precipitate pattern of Fig. 4B, but this cannot be due to the presence of precipitating antibodies against TR-e in the anti-TR-b preparation. The preabsorption of the anti-TR-b antibody with purified flagella did not influence the fluorescence observed with unabsorbed anti-TR-b antibody. No T. pallidum antigen corresponding to TR-b could be demonstrated in T. pallidum sonically treated material with the electroimmunoprecipitation techniques used. Thus, the evidence for an antigen in T. pallidum cross-reacting with TR-b is still indirect.

We have now isolated seven different antigenic components from the Reiter treponema labeled TR-b, TR-c (9), TR-d1 (12), TR-d2 (manuscript in preparation), TR-e (11), TR-o (10), and a lipopolysaccharide (C. Sand Petersen, N. Strandberg Pedersen, and N. H. Axelsen, Anal. Biochem., in press). Except for the lipopolysaccharide, all of the isolated antigens cross-react with antigens of T. pallidum, and these antigens are, therefore, valuable tools in the study of the immune response in syphilis.

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**LITERATURE CITED**


