Monoclonal antibodies to *Treponema pallidum*: recognition of a major polypeptide antigen

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SUMMARY Hybridomas secreting monoclonal antibodies that reacted with a 45,000 dalton surface polypeptide and major immunogen of *T pallidum* were produced. This polypeptide was also found in *T pertenue* but not in *T hyodysenteriae* or *T phagedenis* biotype Reiter.

Introduction

Although recent reports have described biochemical and immunological properties of *Treponema pallidum*, the causative agent of venereal syphilis and non-venereal endemic syphilis or bejel, much remains unknown about the biology of the disease. The humoral immune response appears to play an important part in the pathophysiology of syphilitic infections, and specific surface treponemal proteins have been identified as major immunogens. To further characterise treponemal antigens, we used hybridoma technology to produce antibodies to *T pallidum*. We describe monoclonal antibodies that bind to a 45,000 dalton surface protein of the treponeme.

Materials and methods

**ORGANISMS**

*T pallidum* (Nichols strain) and *T pertenue* (Gautier strain) were provided by the Center for Disease Control, Atlanta, Georgia and were maintained by routine passage in rabbits. Adult New Zealand white rabbits (3-4 kg), obtained locally, were inoculated intratesticularly with 50 × 10^6* T pallidum* treated with 6 mg/kg cortisone acetate (Merck Sharpe and Dohm, West Point, Pennsylvania, USA) on days 3-7 after infection. Peak orchitis occurred at days 10-14 for *T pallidum* and days 24-30 for *T pertenue*. Stock solutions were prepared from infected rabbit testes as described previously. *T phagedenis* biotype Reiter was grown in Spirolate broth supplemented with 0.6% glucose and 10% heat inactivated rabbit serum. *T hyodysenteriae* was a gift of Dr Hank Harris, University of Iowa, Iowa, and was grown in anaerobically reduced media. (*T hyodysenteriae* cultures produce large quantities of hydrogen gas. Care was therefore taken to ventilate these closed cultures to prevent the build up of explosive pressures.)

**RADIOLABELLING OF ORGANISMS**

*T pallidum* harvested from rabbit testes at peak orchitis were concentrated by centrifugation (12,000 × g for 20 minutes) in sterile tubes. Treponemes were resuspended in 2 ml of minimal essential medium without methionine (Gibco, Grand Island, New York, USA) but containing 10% dialysed fetal calf serum and 0.5-1 mCi ^35^S-methionine and were incubated aerobically overnight. Then the treponemes were washed three times in 0.05 mol/l TRIS and 0.2 mol/l sodium chloride (pH 8.0), pelleted, and frozen at −20°C until used.

**IMMUNISATION**

To generate activated mouse spleen cells, freshly extracted and purified *T pallidum* (300 × 10^6 *T pallidum* in 0.5 ml saline) were emulsified 1/1 (vol/vol) in Freund complete adjuvant, and injected intramuscularly (0.1 ml), subcutaneously (0.1 ml), or intraperitoneally (0.3 ml) into BALB/c female mice (3-6 weeks old). On days 7 and 21 the mice were similarly boosted with treponemes emulsified in Freund incomplete adjuvant.

**CELL HYBRIDISATION**

Monoclonal antibodies were produced by modification of the procedure of Oi and Herzenberg. Spleen cells from immunised mice and SP2/0-Ag14
BALB/c myeloma cells were washed separately in Dulbecco's modified Eagle's medium (DME) free of serum (MA Bioproducts, Walkersville, Maryland, USA) and then combined in a ratio of 7:1. The basic method of cell fusion was as described by us earlier. A volume of 50 µl of the fused cell preparation was aliquoted into each well of 96 well microtitre tissue culture plates (Bellco, Vineland, New Jersey, USA) containing 0·1 × 10⁶ normal BALB/c spleen feeder cells in 50 µl of DME-HAT (containing 0·2 mmol/l hypoxanthine, 0·7 mmol/l aminopterin, and 32 mmol/l thymidine) plus glycine. One day after fusion, 100 µl of DME-HAT plus glycine were added to each well. Plates were incubated at 37°C in 7% carbon dioxide for seven to ten days before being screened by an enzyme linked immunosorbent assay (ELISA). Selected hybrid cells were cloned by limiting dilution in 96 well microtitre tissue culture plates containing 0·1 × 10⁶ BALB/c spleen feeder cells/well. Clones were expanded for analysis by ELISA.

ATTACHMENT OF ANTIGEN TO POLYVINYLCHELORIDE MICROTIET PLATES

Individual pellets of treponemes (T pallidum, T pertenue, T hyodysenteriae, and T phagedenis bio-type Reiter) were resuspended in coating buffer and sonicated using six 15 second bursts with 45 second intermittent incubations on ice (Sonifier Cell Disruptor Model W140D, Heat Systems-Ultrasonics, Plainview, New York, USA). The protein concentration of each treponemal preparation was measured by the Lowry method. Aliquots of 100 µl of 1·0 mg/ml treponemal protein in coating buffer were distributed into each well of Immunlon 2 strips (Dynatech, Alexandria, Virginia, USA) and incubated overnight at 4°C. The plates were then filled with 1% bovine serum albumin in phosphate buffered saline (BSA/PBS) and incubated for two hours at 37°C.

Purified T pallidum proteins were isolated from polyacrylamide gels and diluted to 1 mg/ml in coating buffer, aliquoted (50 ng/well) into Immunlon 2 strips, and incubated overnight at 4°C. If not used immediately, plates were washed once with PBS, filled with 1% BSA/PBS, and stored at -20°C.

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

A modified ELISA as previously reported by us was used to detect antibodies to sonicated and purified treponemal proteins.

SERUM SAMPLES

Normal and immune mouse serum was collected by retro-orbital bleeding of BALB/c mice before and after inoculating them with T pallidum. Normal and syphilitic rabbit serum was obtained before or 47 days after infection with T pallidum. Normal and syphilitic human serum was provided by Dr J Jorgenson at Medical Center Hospital, San Antonio, Texas. All serum samples were stored at -20°C before use.

SODIUM DODECYL SULPHATE (SDS) POLYACRYLAMIDE GEL ELECTROPHORESIS

Treponemal proteins were electrophoresed on 7·5% polyacrylamide slab gels in the discontinuous TRIS-glycine system described by Laemmli. After one dimensional electrophoresis, gels were fixed in methanol, acetic acid, and water in a ratio of 45:45:10 and either processed for fluorography with preparations labelled with 35S-methionine or dried immediately and exposed to x-ray film for samples labelled with radioactive iodine (125I).

IODINATION OF PROTEIN A

Using the lactoperoxidase labelling method of Marchalonis et al.100 mg protein A was labelled with 1 mCi radioactive iodine for 30 minutes to give 125I-protein A. After radiolabelling, iodinated protein A was purified from unreactive radioactive iodine and denatured protein A by passage over a 1 ml column of IgG-Sepharose, which was prepared by coupling IgG purified by protein A chromatography to Sepharose 4B activated by cyanogen bromide. Fractions of 1 ml of the PBS eluate were collected until the column background was reduced to less than 5000 counts per minute (cpm)/10 µl aliquot. The bound 125I-protein A was eluted with pH 2·8 glycine hydrochloride and separated from the low molecular weight glycine by subsequent passage over a 0·8 × 25 cm column of Biogel P-10 in PBS.

WESTERN BLOTS

Aliquots (20-40 µg protein) of unlabelled treponemes were solubilised in 0·063 mol/l TRIS containing 2% sodium dodecyl sulphate (SDS), 2% β-mercaptoethanol, and 10% glycerol, and were electrophoresed on a 7·5% polyacrylamide slab gel. The proteins were then electrophoretically transferred for 16 hours at 380 mA to nitrocellulose paper (BioRad, Richmond, California, USA) using the technique of Towbin et al.38 One lane was stained with amido black to show whether both high and low molecular weight proteins were successfully transferred to the nitrocellulose sheets. Non-specific protein binding sites were blocked by incubation in 3% BSA/PBS for six hours. The nitrocellulose strips were then incubated overnight with constant rocking in a 1/50 dilution of antiserum in 3% BSA/PBS or with monoclonal ascites purified antibody. The strips were rinsed in either PBS plus 0·01% SDS or PBS.
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alone and incubated with 3% BSA/PBS containing 125I-protein A. The strips were finally rinsed as before, dried, and exposed to x-ray film (Kodak XR5) with an enhancing screen.39

PURIFICATION OF MONOCLONAL ANTIBODIES ON PROTEIN A SEPHAROSE

Monoclonal antibodies were generated as ascites in pristine primed BALB/c mice.28 The ascites fluid was diluted to 20% in 0.01 mol/l phosphate buffer (pH 7.2) and passed over a 1·5 x 8 cm protein A-Sepharose column. The column was eluted with 0.01 mol/l phosphate buffer (pH 7.2) until the background absorbance (A280) was less than 0.02, followed by elution with 0.1 mol/l glycine (pH 2·8). The fractions containing protein as determined by A280 were pooled and dialysed in a two step process, first against 1000 volumes of pH 5·5 citrate phosphate buffer (0.05 mol/l citric acid, 0·05 mol/l disodium hydrogen phosphate, and 0·145 mol/l sodium chloride; pH adjusted with 6 mol/l sodium hydroxide) and finally against 1000 volumes of PBS. The protein concentrations were measured, and the monoclonal IgG was diluted to 1 g/l with PBS.

Results

Hybridoma culture supernatants were screened for reactivity to whole T pallidum organisms fixed in ethanol. Of about 1200 hybridomas tested, 54 clones bound selectively to T pallidum. To analyse the specificity of the binding sites of these monoclonal antibodies, culture supernatants were screened by ELISA using gel eluted T pallidum proteins33 Treponemal proteins corresponding to specific regions of SDS-polyacrylamide gels were purified and pooled as follows: high molecular weight (HMW) (proteins over 100 000 daltons); P5,6

<p>| TABLE I Reactivity of normal and immune mouse serum and monoclonal antibodies 11F2, 13F3, and 23C9 to T pallidum proteins eluted from sodium dodecyl sulphate polyacrylamide gels |
|---------------------------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Dilution</th>
<th>HMW (OD)</th>
<th>P5,6 (OD)</th>
<th>P1,2,3,4 (OD)</th>
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<tr>
<td>NMS 1:50</td>
<td>0·049 (0·006)</td>
<td>0·059 (0·002)</td>
<td>0·061 (0·004)</td>
</tr>
<tr>
<td>IMS 1:50</td>
<td>0·072 (0·015)</td>
<td>0·375 (0·024)</td>
<td>0·355 (0·115)</td>
</tr>
<tr>
<td>13F3</td>
<td>0·003 (0·005)</td>
<td>0·260 (0·075)</td>
<td>0·355 (0·115)</td>
</tr>
<tr>
<td>23C9</td>
<td>0·002 (0·003)</td>
<td>0·244 (0·033)</td>
<td>0·030 (0·024)</td>
</tr>
</tbody>
</table>

*Mean (SD) of quadruplicate samples. PBS control 0·017. HMW = high molecular weight (>100 000 daltons); P1 (89 500 daltons), P2 (29 500 daltons), P3 (25 500 daltons), P4 (20 000 daltons), P5 (59 000 daltons), P6 (42 500 daltons). For means of other abbreviations see table II.

FIG 1 Western blot analysis of: normal human serum (lane A); syphilitic human serum (lane B); syphilitic rabbit serum (lane C); monoclonal antibody 11F2 (lane D); monoclonal antibody 13F3 (lane E); and monoclonal antibody 23C9 (lane F) against T pallidum antigens.
Western blot analysis of (a) syphilitic rabbit serum, (b) monoclonal antibody 13F3, and (c) normal rabbit serum against: *T. pallidum* (lane 1); *T. pertenue* (lane 2); *T. hyodysenteriae* (lane 3); and *T. phagedenis* biotype Reiter (lane 4) solubilised and electrophoresed on a 7.5% polyacrylamide slab gel.

From the original 54 hybridomas identified in the whole cell ELISA, three hybridomes, (11F3, 13F3, and 23C9) were selected because of their increased reactivity towards the protein pool of P5,6 (table I). Western blot analysis of these monoclonal antibodies confirmed the ELISA results. As shown in fig 1, all three monoclonal antibodies were specific for a 45 000 molecular weight antigen (P6) of *T. pallidum* (lanes D-F), with monoclonal hybridoma 13F3 (lane E) showing the strongest intensity. Human (lane B) and rabbit (lane C) syphilitic serum also showed this major antigen, which was not present in pooled normal human serum (lane A).

The immunological cross reactivity of specific *Treponema* species was then examined. As shown in table II, immune mouse serum was strongly cross reactive with *T. pallidum* and *T. pertenue* antigens and, to a lesser extent, with *T. hyodysenteriae* and *T. phagedenis* indicating shared or cross reactive antigens. Normal mouse serum showed low reactivity to *T. pallidum* and *T. pertenue*, and somewhat increased reactivity to *T. hyodysenteriae* and *T. phagedenis*. To further examine the cross reactivity of these species, Western blot analysis was performed using both normal and syphilitic rabbit serum and monoclonal antibody 13F3. ELISA typing of antibody 13F3 showed the isotype to be IgG2a, x light chain. Only one single heavy and light chain was obtained following SDS-polyacrylamide gel electrophoresis of the antibody. As shown in fig 2, syphilitic

<table>
<thead>
<tr>
<th>Serum</th>
<th>Dilution</th>
<th><em>T. pallidum</em> (OD)</th>
<th><em>T. pertenue</em> (OD)</th>
<th><em>T. hyodysenteriae</em> (OD)</th>
<th><em>T. phagedenis</em> (OD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMS</td>
<td>1:50</td>
<td>0.091 (0.002)</td>
<td>0.058 (0.008)</td>
<td>0.157 (0.019)</td>
<td>0.259 (0.027)</td>
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<tr>
<td>IMS</td>
<td>1:50</td>
<td>1.167 (0.035)</td>
<td>1.121 (0.110)</td>
<td>0.307 (0.146)</td>
<td>0.916 (0.070)</td>
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<tr>
<td></td>
<td>1:100</td>
<td>1.179 (0.072)</td>
<td>0.967 (0.013)</td>
<td>0.314 (0.026)</td>
<td>0.761 (0.031)</td>
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<tr>
<td></td>
<td>1:500</td>
<td>1.825 (0.086)</td>
<td>0.763 (0.064)</td>
<td>0.059 (0.012)</td>
<td>0.450 (0.004)</td>
</tr>
</tbody>
</table>

*Mean (SD) of quadruplicate samples. PBS control = 0.017.
†Treponemes were prepared and sonicated as described in Materials and methods. Treponemal proteins were coated on to microtitre strips at a concentration of 1 µg protein per well.
OD = optical density at 405 nm; NMS = normal mouse serum; IMS = immune mouse serum.
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rabbit serum (a) showed cross reactive antigens among all four treponemal species. Normal rabbit serum (c) did not react with *T pallidum* (lane 1) or *T pertenue* (lane 2) but low level reaction occurred with *T hyodysenteriae* (lane 3) and *T phagedenis* biotype Reiter (lane 4). Monoclonal antibody 13F3(b) was highly selective for the 45,000 dalton protein antigen (P6) found in *T pallidum* and *T pertenue*. No cross reactive protein was shown by 13F3 in *T hyodysenteriae* or *T phagedenis*.

**Discussion**

A group of 54 monoclonal antibodies reactive with *T pallidum* were produced. Three hybridoma clones were selected for further study because of their unique specificity for a 45,000 dalton molecular weight polypeptide, P6. Antibodies to this protein have been shown to appear early (day 10) in rabbits experimentally infected with *T pallidum*. This protein also exhibits high immunogenicity in infected rabbits and man.

Monoclonal antibody 13F3 was purified by protein A-Sepharose chromatography and used to examine cross reactivity among four species of treponemes (fig 2). In contrast to polyclonal antiserum raised against *T pallidum*, which shows strong reactivity with all four species of treponemes, monoclonal 13F3 reacted only with *T pallidum* and *T pertenue*. These data further emphasise the close relation between these two treponemes and reinforce the biological differences between the treponemes pathogenic and non-pathogenic to man.

The immune response to syphilis is extremely complex and includes both humoral and cell-mediated immunity. Monoclonal antibody probes offer the opportunity to evaluate better the role of specific treponemal antigens in the pathophysiology of the disease. The availability of monoclonal probes also permits further biochemical and immunohistochemical dissection of important antigens and enables the development of rational diagnostic reagents and vaccines.

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**References**


