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 pertenu* but not in *T hyodysenteriae* or *T phagedenis* biotype Reiter.

Introduction

Although recent reports have described biochemical and immunological properties of *Treponema pallidum*,¹⁻²⁵ 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.^{3 4 6-8}

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 pertenu* (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 treponemes and 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 pertenu*. Stock solutions were prepared from infected rabbit testes as described previously.² *T phagedenis* biotype Reiter was grown in Spirolate broth supplemented

with 0.6% glucose and 10% heat inactivated rabbit serum.²⁶ *T hyodysenteriae* was a gift of Dr Hank Harris, University of Iowa, Iowa, and was grown in anaerobically reduced media.²⁷ (*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 ($12\,000 \times g$ 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 ³⁵S-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.²⁸ Spleen cells from immunised mice and SP2/0-Ag14

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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.²⁹ A volume of 50 μ l of the fused cell preparation was aliquoted into each well of 96 well microtitre tissue culture plates (Bellco, Vineland, New Jersey, USA) containing 0.1×10^6 normal BALB/c spleen feeder cells in 50 μ l of DME-HAT (containing 0.2 mmol/l hypoxanthine, 0.7 mmol/l aminopterin, and 32 mmol/l thymidine)³⁰ plus glycine. One day after fusion, 100 μ l of DME-HAT plus glycine were added to each well. Plates were incubated at 37°C in 7% carbon dioxide for seven to 10 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 0.1×10^6 BALB/c spleen feeder cells/well. Clones were expanded for analysis by ELISA.

ATTACHMENT OF ANTIGEN TO POLYVINYLCHLORIDE MICROTITRE PLATES
Individual pellets of treponemes (*T pallidum*, *T pertenue*, *T hyodysenteriae*, and *T phagedenis* biotype Reiter) were resuspended in coating buffer³¹ 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.³² Aliquots of 100 μ l of 1.0 mg/ml treponemal protein in coating buffer were distributed into each well of Immulon 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 gels³³ and diluted to 1 mg/ml in coating buffer,³¹ aliquoted (50 ng/well) into Immulon 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^{29 33} 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.³⁴ 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-methionine³⁵ 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*.³⁶ 100 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.³⁷ 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 μ l 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 μ g protein) of unlabelled treponemes were solubilised in 0.063 mol/l TRIS containing 2% sodium dodecyl sulphate (SDS), 2% β -mercapto-ethanol, 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

alone and incubated with 3% BSA/PBS containing ^{125}I -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 1.5×8 cm protein A-Sepharose column. The column was eluted with 0.01 mol/l phosphate buffer (pH 7.2) until the background absorbance (A_{280}) was less than 0.02, followed by elution with 0.1 mol/l glycine (pH 2.8). The fractions containing protein as determined by A_{280} 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* proteins.³³ 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

(59 000 and 42 500); and P1,2,3,4 (89 500, 29 500, 25 500, 20 000 daltons). These protein designations (P1-P6) are consistent with previous reports.^{3 4 6-8} As shown in table I, immune mouse serum (IMS) obtained just before killing for the hybridoma fusion reacted predominantly with protein pools P1-4 and P5,6; there was low reactivity towards the high molecular weight antigen group. Normal mouse serum (NMS) showed poor reactivity to all antigen groups examined.

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.

TABLE 1 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

	Dilution	Mean (SD)* reactivity to <i>T pallidum</i> proteins		
		HMW (OD)	P5,6 (OD)	P1,2,3,4 (OD)
NMS	1:50	0.049 (0.006)	0.059 (0.002)	0.061 (0.004)
IMS	1:50	0.072 (0.015)	0.375 (0.024)	0.355 (0.115)
	1:100	0.033 (0.004)	0.357 (0.021)	0.237 (0.097)
11F ₂	1:100	0.005 (0.034)	0.138 (0.047)	0.034 (0.019)
13F ₃	1:100	0.003 (0.005)	0.260 (0.075)	0.001 (0.004)
23C ₉	1:100	0.002 (0.003)	0.244 (0.033)	0.030 (0.024)

* Mean (SD) of quadruplicate samples. PBS control ≤ 0.017 . HMW = heavy 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 meanings of other abbreviations see table II.

FIG 2 Western blot analysis of (a) syphilitic rabbit serum, (b) monoclonal antibody 13F₃, and (c) normal rabbit serum against: *T pallidum* (lane 1); *T pertenu* (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 hybridomas, (11F₂, 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 pertenu* 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 pertenu*, 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, κ 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 II Reactivity of normal and immune mouse serum to various treponemal species

Serum	Dilution	Mean (SD)* reactivity to sonicated treponemal antigens†			
		<i>T pallidum</i> (OD)	<i>T pertenu</i> (OD)	<i>T hyodysenteriae</i> (OD)	<i>T phagedenis</i> (OD)
NMS	1:50	0.091 (0.002)	0.058 (0.008)	0.157 (0.019)	0.259 (0.027)
IMS	1:50	1.167 (0.035)	1.121 (0.110)	0.507 (0.146)	0.916 (0.070)
	1:100	1.179 (0.072)	0.967 (0.018)	0.314 (0.026)	0.761 (0.031)
	1:500	1.825 (0.086)	0.763 (0.064)	0.059 (0.012)	0.450 (0.004)

* 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 μ g protein per well.

OD = optical density at 405 nm; NMS = normal mouse serum; IMS = immune mouse serum.

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 pertenu* (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*³ and *T pertenu*.⁹ 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*.¹⁵ This protein also exhibits high immunogenicity in infected rabbits and man.^{3 4 6}

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 pertenu*. These data further emphasise the close relation between these two treponemes,^{9 40} 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.^{9 41 42} The availability of monoclonal probes also permits further biochemical and immunochemical dissection of important antigens and enables the development of rational diagnostic reagents and vaccines.

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