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Enzyme-linked immunosorbent assay for the detection of serum antibody to outer membrane proteins of Treponema pallidum

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SUMMARY A highly sensitive enzyme-linked immunosorbent assay was used for the analysis of serum IgG reactivity against specific immunogenic *Treponema pallidum* proteins. Outer membrane treponemal proteins purified by preparative SDS-polyacrylamide gel electrophoresis were used as antigenic probes at concentrations as low as 100 ng per ml (5 ng per well in microtitre plates). Detection of anti-treponemal antibody was possible using rabbit syphilitic sera diluted to 1/10 000. The sensitivity of the assay was equal to or greater than that detected by radioimmunoprecipitation combined with gel electrophoresis and fluorography techniques and was capable of monitoring host IgG responses throughout the progress of the disease.

Introduction

Immunological characterisation of the host response to *Treponema pallidum* infection has identified several outer membrane treponemal proteins as major immunogens.[1-4] Serum IgG antibodies against these *T pallidum* antigens in patients with syphilis and in experimentally infected rabbits have been detected by radioimmunoprecipitation combined with sodium dodecylsulphate-polyacrylamide gel electrophoresis and autoradiography-fluorography techniques.[3] These data indicated a predictable pattern for early and prolonged host humoral response to specific *T pallidum* polypeptides.

We report a simple, rapid, and highly sensitive microassay which circumvented standard radioimmunoassays, crossed-immunoelectrophoresis procedures, and other classical antigenically-undefined serological tests.[5-9]

Materials and methods

TREPONEMA PALLIDUM ORGANISMS

Treponema pallidum (Nichols strain) organisms were passaged in testes of New Zealand white male rabbits (3-3·5 kg). The rabbits were maintained at 16-18°C in isolation cubicles before and during treponemal infection. Isolated treponemes were preserved in 10% dimethyl sulphoxide in liquid nitrogen before intratesticular inoculation of the rabbits.[10] Treponemes were extracted and purified according to procedures described.[1-2]

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RADIOIMMUNOPRECIPITATION ASSAY

These assays were performed as described.[3]

T PALLIDUM PROTEINS FROM ACRYLAMIDE GELS

Specific treponemal proteins previously labelled protein 1 (P1; 89 500 daltons), P2 (29·5 K), P3 (25·5 K), P4 (20 K), P5 (59 K), and P6 (42·5 K) were purified by preparative electrophoresis on sodium dodecylsulphate (SDS)-polyacrylamide (7·5%) (PAGE) slab gels as described for *T pallidum*.[1] The location of appropriate protein bands was determined by Coomassie brilliant blue (Sigma Chemical Co, St Louis, MO, USA) staining of a small longitudinal section of the gel and then by direct correlation between these profiles and matched gel patterns of [^{35}S]methionine intrinsically-labelled *T pallidum* proteins. Corresponding unstained protein bands in the preparative gels were sliced, macerated, and gently shaken in H_2O for 48-60 hours at 4°C.[11] Except for P1, the remaining protein bands were mixed in combinations of P2P3 and P4P5P6.

The eluted protein suspension was centrifuged at 17 000 × *g* for 20 minutes to pellet acrylamide fragments. Supernatants were lyophilised and reconstituted in 1-2 ml H_2O. The eluted preparations were re-examined by SDS-PAGE using 7·5% acrylamide slab gels to establish their purity. Protein concentrations were estimated by the Bradford technique[12] and by comparison of the intensity of stained bands of purified protein to standard bovine serum albumin (BSA; Sigma).

ANTIGEN ATTACHMENT TO POLYVINYL-CHLORIDE MICROTITRE PLATES

Freshly extracted *T pallidum* organisms were purified by Methocel (Dow Chemical Co, Midland, MI)-Hypaque (Winthrop Laboratories, New York) gradient centrifugation as described.[1] Treponemal suspensions containing approximately 3×10^8 organisms per ml were washed once using phosphate-buffered saline (PBS; 137 mmol/l NaCl, 2·7 mmol/l KCl, 4·6 mmol/l Na_2HPO_4, and 1·5 mmol/l KH_2PO_4, pH 7·2) and centrifuged for 10 minutes at 17 500 × *g*. Pellets were resuspended to $3·5 \times 10^7$ treponemes per ml PBS.[13 14] Fifty microlitres were distributed into each well of 96-well PVC microtitre plates (Dynatech Laboratories, Alexandria, VA, USA), and suspensions dried in air at 37°C. Then 50 µl of 95% ethanol were added to each well, and plates were dried at 37°C and stored under desiccant at 4°C until used.

Purified *T pallidum* proteins were diluted to 1 µg/µl in carbonate buffer,[15] aliquoted (50 ng/well) on to 96-well microtitre plates, and incubated overnight at 4°C. If not used immediately, plates were washed once with PBS, filled with PBS-1% BSA and stored at −20°C.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

A modified ELISA technique[15] was used for detecting antibodies to both whole *T pallidum* organisms and extracted treponemal proteins. Briefly, after antigen coating of PVC microtitre plates wells filled with PBS supplemented with 1% BSA were incubated for a minimum of two hours at 37°C. Plates were then washed three times with PBS, and 50 µl of test serum diluted with PBS-1% BSA were added to the designated wells. After incubation for one hour at 37°C the plates were washed four times with PBS. Fifty-microlitre aliquots of alkaline phosphatase conjugated goat anti-rabbit IgG (Miles Laboratories, Elkhart, IN, USA) diluted 1/3000 in PBS-1% BSA were then added to each well. Plates were incubated for one hour at 37°C followed by sequential washing with PBS (three times) and distilled H_2O (twice). Finally, 50 µl of 1 mg/ml disodium p-nitrophenyl phosphate (Sigma) prepared in diethanolamine buffer[15] were added to each well, and the plates incubated for 30 minutes at 37°C. Antibody reactivity was measured by optical density readings at 405 nm using a MicroElisa Reader (Dynatech).

SERA

Serum samples were collected from the ear vein of rabbits before and at various intervals after infection.[3] All sera were stored at −70°C before use.

Results

ANTIGEN TITRATION OF PURIFIED *T PALLIDUM* PROTEINS

To determine the sensitivity of the ELISA using gel-eluted treponemal proteins preparations of previously characterised proteins P1, P2P3, and P4P5P6[1-4] were attached to wells of microtitre plates and used as probes for antibody reactivity. Antigen titration was performed using pre-bled normal rabbit serum (NRS) and rabbit syphilitic serum (RSS) obtained at day 48 post-inoculation from *T pallidum*-challenged animals.[2 3] When diluted 1/100 RSS detected as little as 0·1 µg/ml of antigens P2P3 and P4P5P6 (fig 1). At the same serum dilution NRS produced only a minimal background response to all antigens regardless of concentration. Nevertheless 1·0 µg/ml was routinely used to allow a response which was 95% of the maximum ELISA reactivity detected with RSS to P2P3. This concentration additionally resulted in a value at least 30 times greater than that of NRS. Although RSS reacted with all three antigen preparations, the response to P2P3 at 1·0 µg/ml was about 18 times greater than that to P1 and three to four times the response to P4P5P6. These data confirm that the predominant IgG response by day 48 RSS is directed against P2P3 antigens as was previously shown by radioimmunoprecipitation and gel electrophoresis,[3] (fig 2).

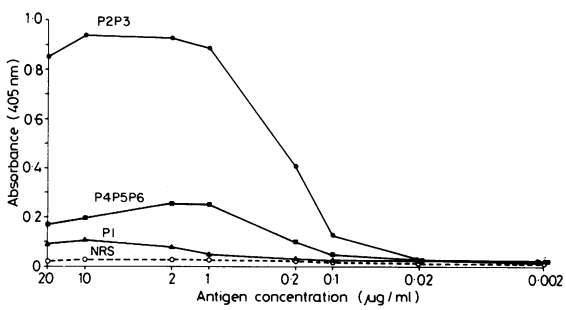

FIG 1 *Comparative sensitivity of purified treponemal protein preparations by ELISA. Standard deviations were <0·05 for all values shown.*

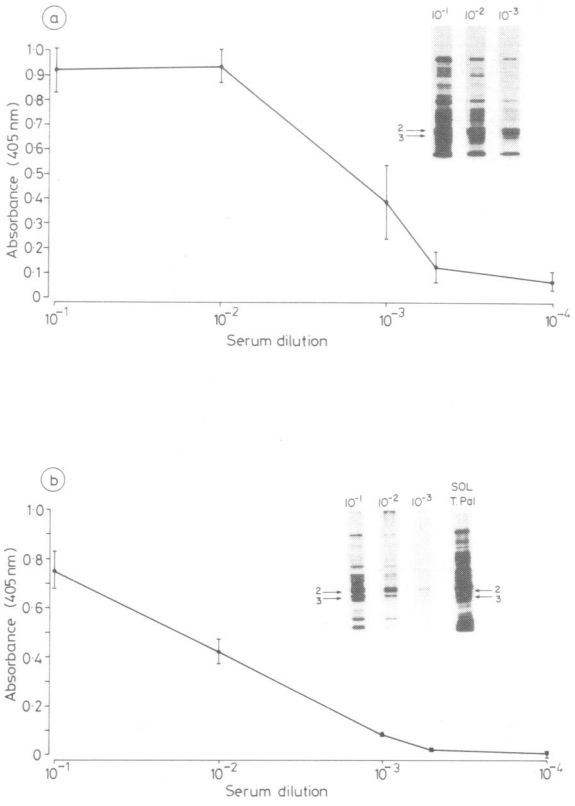

FIG 2 *Titration of representative syphilitic rabbit serum IgG reactive against purified P2P3 at day 48 (a) and day 160 (b) after T pallidum infection. Corresponding radioimmunoprecipitation fluorograms of [^{35}S]methionine-labelled, detergent-solubilised treponemal proteins reactive with the same serum are shown in the top right corner. The SOL T Pal profile (b) represents the profile of total treponemal proteins. Proteins previously characterised as P2P3 are denoted by arrows.*

ANTIBODY TITRATION OF RSS TO PURIFIED P2P3

The kinetics of the IgG response of rabbits infected with *T pallidum* was examined to determine antibody sensitivity of the ELISA to P2P3 at 10, 48, and 216 days after challenge. Antibodies against P2P3 antigens were detectable at each stage of infection (table I). To establish a consistent method for determining serum titration endpoints values greater than 20% of NRS were routinely considered positive. Of special interest was the high sensitivity of the assay. All test sera were positive except for the 1/10 000 dilution of sera at day 216.

SENSITIVITY OF THE ELISA COMPARED WITH RADIOIMMUNOPRECIPITATION

Radioimmunoprecipitation used in combination with gel electrophoresis-fluorography is a highly accurate technique for detecting IgG against *T pallidum* proteins.[2-4] It was important, therefore, to compare the ELISA with RIP using RSS obtained at varying times after infection. These sera have been previously characterised for reactivity to specific treponemal proteins.[3]

The ELISA detected a pronounced IgG response to P2P3 (50 ng protein/well) even at the 1/1000 (10^{-3}) dilution of serum obtained on day 10 after challenge (table I). These data are consistent with previous observations of serum IgG antibody directed predominantly against P2P3 early in infected animals.[2 3] At day 48 after inoculation serum diluted to 10^{-4} still showed detectable antibody against P2P3 (fig 2a). Corresponding RIP data indicated the presence of IgG to P2P3 up to the 10^{-3} dilution.

A direct correlation between the ELISA and RIP is shown in fig 2b. Based on the RIP profile RSS at day 160 was primarily reactive to proteins P2P3 at a serum dilution of 10^{-2} and to P2 with serum diluted 10^{-3}. Similarly, the ELISA measured significant reactivity against P2P3 at the 10^{-1}, 10^{-2}, and 10^{-3} dilutions of RSS. Only low level absorbance was

TABLE I *Antibody titration to purified P2P3 T pallidum proteins*

	Absorbance (405nm) (days after inoculation):		
Serum dilution	*10 (mean ± SD)**	*48 (mean ± SD)**	*216 (mean ± SD)**
1/10	0·830 ± 0	0·909 ± 0·021	0·508 ± 0·028
1/100	0·598 ± 0·005	0·763 ± 0·023	0·254 ± 0·018
1/500	0·247 ± 0·011	0·712 ± 0·008	0·093 ± 0·001
1/1000	0·153 ± 0·003	0·336 ± 0·011	0·047 ± 0·008
1/5000	0·024 ± 0·001	0·039 ± 0·004	0·008 ± 0·003
1/10 000	0·011 ± 0	0·019 ± 0·001	0·001 ± 0

* Of duplicate samples from three different experiments. NRS at a 1/100 dilution gave values of 0·020 ± 0. At further dilutions no NRS response was detectable (<0·001).

TABLE II *Comparative reactivity of rabbit sera IgG to whole T pallidum and specific P2P3 protein preparations*

Experiment	Antigen	Serum dilution	Absorbance (405nm) (days after inoculation): 16 mean ± SD)*	64 (mean ± SD)*	160 (mean ± SD)*
1	P2P3 preparation	1/10	0·864 ± 0·003	0·856 ± 0·044	0·809 ± 0·030
		1/100	0·756 ± 0·021	0·959 ± 0·081	0·460 ± 0·028
		1/1000	0·288 ± 0·006	0·513 ± 0·007	0·091 ± 0·005
		1/5000	0·063 ± 0·002	0·178 ± 0·002	0·020 ± 0·003
		1/10 000	0·030 ± 0·009	0·103 ± 0·008	0·014 ± 0·004
	Whole *T pallidum*	1/10	0·519 ± 0·016	0·614 ± 0·110	0·623 ± 0·213
		1/100	0·246 ± 0·007	0·347 ± 0·040	0·584 ± 0·060
		1/1000	0·088 ± 0·008	0·138 ± 0·028	0·136 ± 0·084
		1/5000	0·042 ± 0·015	0·105 ± 0·024	0·051 ± 0·012
		1/10 000	0·037 ± 0·022	0·068 ± 0·008	0·009 ± 0·013
2	P2P3 preparation	1/10	0·950 ± 0·015	0·986 ± 0·094	0·695 ± 0·053
		1/100	0·827 ± 0·128	0·908 ± 0·068	0·380 ± 0·024
		1/1000	0·224 ± 0·006	0·258 ± 0·030	0·077 ± 0·006
		1/5000	0·051 ± 0·006	0·068 ± 0·006	0·020 ± 0·002
		1/10 000	0·026 ± 0·003	0·037 ± 0	0·013 ± 0·005
	Whole *T pallidum*	1/10	0·907 ± 0	0·788 ± 0·028	0·643 ± 0·040
		1/100	0·883 ± 0·141	0·827 ± 0·013	0·352 ± 0·139
		1/1000	0·365 ± 0·05	0·246 ± 0·06	0·076 ± 0·011
		1/5000	0·076 ± 0·006	0·025 ± 0·021	0·026 ± 0·036
		1/10 000	0·025 ± 0·011	0·018 ± 0	0·013 ± 0·001

* Of duplicate samples from three different experiments

detectable with NRS throughout these ELISA experiments indicating the absence of reactive antibody.

COMPARISON OF ELISA SENSITIVITY WITH P2P3 VERSUS WHOLE *T PALLIDUM*

Because whole organisms have been used as sources of antigen for treponemal serodiagnostic assays[13 14] it was of interest to compare ELISA titres of RSS between intact *T pallidum* and P2P3 protein preparations. Representative test samples of RSS at days 16, 64, and 160 after infection were examined. As shown in table II antitreponemal IgG reactivity was more readily detected using purified P2P3 than whole *T pallidum*. Additionally, decreased variability was shown using P2P3. In repeated experiments serum titrations based on P2P3 reactivity were consistently equal to or greater than whole treponemal organisms.

Discussion

In the present study an ELISA was developed for the analysis of serum IgG against immunogenic and biologically important *Treponema pallidum* proteins. Recent immunochemical dissection of virulent *T pallidum* has established that certain treponemal proteins play key roles during the ontogeny of the host immune response to infection.[2-4] Radioimmunoassay-gel electrophoresis techniques, together with surface iodination procedures, show that outer membrane proteins P1 to P6 have high immunogenicity in experimentally infected rabbits and humans with syphilis.[2-4] Proteins P1, P2, and P3 have also been implicated as putative receptor-binding proteins in the attachment of *T pallidum* to host cell surfaces.[2-4] Acrylamide gel purification and elution of these treponemal proteins has provided selective antigenic probes for the serological diagnosis of treponemal infection. Sensitivity of this microassay was confirmed by detection of specific binding of serum IgG to as little as 5 ng per well of purified antigen (fig 1). Each preparative electrophoresis-protein elution experiment required the harvesting of *T pallidum* from 6 to 8 rabbits. Approximately 100 to 200 μg of specific treponemal protein could be obtained providing sufficient antigen to coat a minimum of 20 microtitre plates or 2000 wells. Reproducible results were obtained using antigen-coated plates stored over a period of several months.

The sensitivity of the ELISA was equivalent to or better than the RIP, avoiding the need to use radioactive reagents and time-consuming procedures. In addition, the use of purified treponemal proteins afforded a clear advantage to whole *T pallidum* organisms since identification of specific antigen-antibody complexes and high reproducibility of data were possible. The technical problems of treponemal clumping, masking of *T pallidum* antigens with host components,[1] and biological variations in treponemal populations[1] were avoided.

The ELISA has been successfully used for analysis of a wide variety of bacterial, viral, and cellular antigens.[15-18] It is an efficient, simple, and relatively inexpensive assay, which is superior to other serological tests when highly immunogenic and purified antigenic indicators are used. Stability and long-term storage of reagents allow the assay to be particularly suitable for large-scale screening. As reported here, the technique resulted in a high degree of specificity and sensitivity. The rationale for selecting appropriate *T pallidum* immunogens such as P2P3 was a direct result of recent biochemical and immunological data.[1-4] Characterisation of the kinetics of the humoral response to *T pallidum* infection indicates that antibody against proteins P2P3 may correlate with the development of host immunity. In addition, detection of antibodies to these proteins in human syphilis[3 4] and yaws[4] further extends the application and relevance of this immunodiagnostic microassay.

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