Antigenic contents of *Treponema pallidum* preparations

SUSAN M WOS*, AND KONRAD WICHER*†

From the *Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, New York, and the †Department of Microbiology, School of Medicine, State University of New York at Buffalo, Buffalo, New York, United States of America

SUMMARY In investigations of syphilis various *Treponema pallidum* antigens are used to study the immune responses of naturally or experimentally infected hosts. In the past these antigen preparations have rarely been examined for their antigenic contents and activity. In the present study, supernatant, sediment, and solubilised preparations of *T pallidum* Nichols strain (20×10^9 organisms/ml) and *T phagedenis* biotype Reiter were examined by modified counterimmunoelectrophoresis and immunoblotting for their antigenic contents. No *T pallidum* antigen was seen in the supernatant fraction, which contained cross reacting (Reiter) antigens and rabbit serum proteins. The remaining *T pallidum* preparations contained *T pallidum* antigens, cross reacting treponemal (Reiter) antigens, and rabbit serum proteins.

These findings suggest that *T pallidum* preparations should be examined qualitatively and quantitatively before they are used for monitoring immune responses and interpreting data. Technology for these examinations is available.

Introduction

Eighty years of research into syphilis has contributed greatly to the diagnosis and treatment of the disease but little to our understanding of the causative agent, *Treponema pallidum*, and the immunopathology of the infected host. The major reason for this lack of progress is the complexity of the organism and the inability so far to culture it in vitro in a cell free medium. Instead, the treponemes used for various diagnostic or research purposes are propagated in rabbit testes. Separating treponemal antigen from host proteins and isolating and purifying species specific antigens is extremely difficult.

In studies during years of research the immune responses of hosts infected with *T pallidum* have been assessed by using various *T pallidum* preparations, including intact organisms¹⁻³ and sonicated,^{4,5} sonicated and centrifuged,⁶ or cryolysed, sonicated, and centrifuged material.⁷ Very rarely were these antigen preparations examined for their antigenic activity.⁸ To our knowledge, there have been no published reports relating to immune response studies in which the concentration of specific *T pallidum* in the

assay antigen has been measured. The varying concentrations, purity, and specificity of these preparations could account in part for the discrepancies between the reported data.

Future investigations on measuring immune responses to infection with *T pallidum* depend on the answers to following key questions: how much of the specific antigen is present in a given *T pallidum* preparation, how important is it to measure specific responses, and will they differ from those obtained with the complex crude treponemal antigen?

Material and methods

REAGENTS

T pallidum Nichols is maintained in our laboratory by intratesticular passage in adult Nys (Flemish Giant) rabbits,⁹ which were used to obtain *T pallidum* preparations and antisera for the present study. Intratesticular infection was accomplished by inoculation of 20×10^6 organisms per testis. At the time of orchitis, nine to 12 days after infection, the organisms were extracted repeatedly from the orchitic tissue into phosphate buffered saline (PBS), pH 7.2 (20 ml/testis). The suspensions were filtered through polycarbonate filters with $0.8 \mu\text{m}$ sized pores (Nucleopore Corporation, Pleasanton, California, USA), and the organisms were pelleted by centrifugation at $39\,100 \times g$ for one hour, followed by three washes in excess PBS. A suspension of 20×10^9

Address for reprints: Dr Konrad Wicher, Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, New York 12201, USA

Accepted for publication 12 September 1985

organisms/ml was sonicated (MSE Incorporated, Westlake, Ohio, USA) for six to eight cycles of one minute each until all the organisms were disrupted, as confirmed by dark field microscopy. The sonicate was centrifuged at $39\,100\times g$ for one hour. The supernatant was removed, and the sediment was resuspended to the original volume with PBS.

Solubilised *T pallidum* antigen was prepared from a filtered suspension containing 20×10^9 organisms/ml in 10 mmol/l tromethamine (TRIS) and 1.5 mmol/l disodium ethylenediamine tetraacetate, pH 7.2. The organisms were sonicated as described above. Sodium-N-lauryl sarcosine was added to a final concentration of 2%, and the solution was incubated at 37°C for 30 minutes with constant stirring.

Treponema phagedenis biotype Reiter was grown in spirochaetal medium with 10% rabbit serum.¹⁰ Washed organisms were adjusted to 20×10^9 organisms/ml and used to prepare supernatant, sediment, and solubilised fractions as for *T pallidum*.

Normal rabbit serum and normal rabbit testicular fluid were used as controls. Normal rabbit testicular fluid was prepared from five rabbits by aseptically removing the testes, cutting each in half, and centrifuging each half at $12\,000\times g$ for 30 minutes. The transudate was centrifuged at $39\,000\times g$ for one hour. All antigen preparations were aliquoted and stored at -70°C until used.

Antiserum to *T pallidum* was obtained from eight rabbits by nine intravenous injections with virulent *T pallidum* Nichols strain (20 to 50×10^6 organisms) during a three month period. An IgG fraction, prepared by diethylaminoethanol (DEAE) chromatography,¹¹ was adsorbed with glutaraldehyde polymerised normal rabbit serum and normal rabbit testicular fluid,¹² followed by adsorption with cyanogen bromide activated Sepharose gel coupled with sonicated *T phagedenis* Reiter (8 mg protein/ml gel).

Rabbit antiserum to *T phagedenis* Reiter was prepared by immunising the animals with washed organisms (10^{10} /ml) emulsified in complete Freund's adjuvant. The animals received four bimonthly intradermal injections at multiple sites (eight to ten). The antiserum was adsorbed with glutaraldehyde polymerised normal rabbit serum.

Titres of antibodies to *T pallidum* and *T phagedenis* biotype Reiter and the effects of adsorptions were measured by the fluorescent antibody test or by modified counterimmunoelectrophoresis.

Antisera to host proteins were prepared by immunising guinea pigs with normal rabbit testicular fluid or normal rabbit serum preparations emulsified in complete Freund's adjuvant. Five intradermal injections (0.5 g/l) were given over six months. The titres were measured by counterimmunoelectrophoresis.

Counterimmunoelectrophoresis

We used a two step modification of counterimmunoelectrophoresis (Dr Victoria Wicher, unpublished observation). A 1.5 mm thick gel slab was prepared by pouring 15 ml of 1% agarose prepared in barbital buffer (pH 8.6) on to a glass plate. Pairs of wells 3 mm in diameter were punched 6 mm apart in a row down the plate. The anodal wells were filled with 10 µl of antiserum and electrophoresed at 250 V for 20 minutes. The cathodal wells were then filled with 10 µl of the appropriate antigen and electrophoresed for an additional 30 minutes. The slides were washed for three days with frequent changes of PBS and soaked overnight in half strength barbital buffer to equilibrate the agarose. After removing fluid from the wells, an immunoglobulin fraction (10 g/l) of antirabbit or antigoat antiserum was added to the anodal wells. The gels were electrophoresed under the same conditions as described previously. The plates were then washed and stained and the results read.

Fluorescent antibody test

We performed the test as described previously.¹³ In general, heat inactivated serum samples were applied on Treposlides (Beckman Instruments, Fullerton, California, USA), incubated at 37°C for 30 minutes, and washed. Antiserum conjugated with fluorescein isothiocyanate (fluorescein to protein ratio 2:8) was then applied and after being washed the slides were examined under a Nikon Optiphot microscope.

Western blot technique

We examined supernatant and sediment fractions of various concentrations of *T pallidum* as described previously.¹⁴ The treponemal preparations were electrophoresed on a 10% polyacrylamide slab gel in a TRIS-glycine system, as described by Laemmli.¹⁵ Proteins were electrophoretically transferred on to nitrocellulose paper¹⁶ in a Trans-Blot Cell (Bio Rad Laboratories, Richmond, California, USA) with TRIS-glycine-methanol buffer at 8 V/cm for three hours at 4°C. The nitrocellulose blots were probed for two hours with antiserum to *T pallidum* adsorbed with normal rabbit testicular fluid, normal rabbit serum, and *Treponema phagedenis* Reiter.

Results

The minimum concentrations of antigens detected by the modified counterimmunoelectrophoresis were: *T pallidum*, 60 µg/ml; *T phagedenis* Reiter, 2 µg/ml; and normal rabbit serum, 0.2 µg/ml.

Both *T pallidum* and *T phagedenis* Reiter preparations were assayed for treponemal and host antigens by counterimmunoelectrophoresis. The table summarises the results. Adsorption of *T pallidum* antiserum with *T phagedenis* Reiter eliminated

TABLE Detection of antigens by counterimmunoelectrophoresis

Antisera	Reaction with:					
	<i>Treponema pallidum</i> Nichols strain			<i>Treponema phagedenis</i> biotype Reiter		
	Supernatant	Sediment	Solubilised	Supernatant	Sediment	Solubilised
<i>T. pallidum</i> Nichols	-	+	+	-	-	-
<i>T. phagedenis</i> Reiter	+	+	+	+	+	+
Normal rabbit serum	+	+	+	+	+	+
Normal rabbit testicular fluid	-	-	-	-	-	-

FIGURE Detection of *Treponema pallidum* Nichols antigens by western blot technique. Preparations containing 0.1×10^9 to 20×10^9 *T. pallidum* were processed to obtain sediment and supernatant fractions, which were reacted with adsorbed antiserum to *T. pallidum*. (*T. pallidum* antigens were detected in three sediments (1×10^9 to 20×10^9) but not in the supernatants.)

detectable cross reactivity to this organism. The adsorbed antiserum detected *T. pallidum* antigens only in *T. pallidum* sediment and solubilised preparations. No *T. pallidum* antigens were detected in *T. pallidum* supernatant, which contained cross reacting treponemal (Reiter) antigens and rabbit serum proteins. No testicular proteins were detected in any *T. pallidum* preparation. All three preparations of *T. phagedenis* Reiter contained treponemal antigens and rabbit serum proteins.

To confirm the absence of *T. pallidum* antigens in *T. pallidum* supernatant, this and *T. pallidum* sediment preparations of various concentrations of *T. pallidum* were examined by the western blot technique with the adsorbed antiserum to *T. pallidum*. No *T. pallidum* antigens were detected in *T. pallidum* supernatant, even at the highest concentration of *T. pallidum* (figure). The *T. pallidum* sediment preparation contained *T. pallidum* antigen, which decreased with decreasing concentrations of *T. pallidum*. The molecular masses of the reactive peptides in *T. pallidum* ranged from 46 to 18 kilodaltons. Peptides with molecular masses greater than 46 kilodaltons, which had been reported previously in solubilised *T. pallidum* preparations,^{14 17 18} were absent from *T. pallidum* sediment. Our antiserum, however, had been absorbed to the point of not reacting by counter-immunoelectrophoresis with cross reactive Reiter antigens.

Discussion

No information is available on the sensitivity of the western blot technique. About 10^8 to 10^{10} *T. pallidum* micro-organisms are most commonly used.^{14 17 18} The solid phase immunoblot assay, however, which is a modification of the colony blot radioimmunoassay, showed positive reactions with as few as 1000 *T. pallidum* organisms when used to analyse monoclonal antibodies.¹⁹

The sensitivity of any immunological test using the *T. pallidum* system is influenced by the presumably weak avidity of the antibodies and the degree of specificity of the antiserum. Before adsorption our antiserum to *T. pallidum* had a fluorescent antibody titre of 1/10 000, which decreased to 1/8000 after repeated

adsorptions with a suspension of intact washed *Treponema phagedenis* Reiter (10^{10} organisms). After two further adsorptions with sonicated Reiter organisms coupled to cyanogen bromide activated Sepharose the titre decreased to 1/4000, which indicates that adsorption with the intact organism removes only part of the cross reacting antibodies. This antiserum no longer reacted with Reiter antigens or with normal rabbit serum by counterimmunoelectrophoresis.

The results of the present study indicate that *T pallidum* supernatant did not contain measurable *T pallidum* antigen, even when the original concentration of the suspension was 20×10^9 organisms/ml. Even if *T pallidum* antigen is present in minute amounts in the supernatant, it must be overwhelmed in the competition with cross reacting treponemal antigens and host serum proteins.

We therefore conclude that the *T pallidum* supernatant is not a suitable reagent for measuring the immune response in hosts infected with *T pallidum*. Moreover the *T pallidum* sediment fraction obtained from a suspension of 10^8 *T pallidum*/ml does not have a measurable amount of treponemal antigen. The techniques of the immunoblotting system are available and should be used in qualitative analysis of treponemal reagents.

Strictly defined specific antigens should be used to re-examine the kinetics of the humoral and cellular response of hosts infected with *T pallidum*.

References

- Musher DM, Schell RF, Jones RH, Jones AM. Lymphocyte transformation in syphilis: in vitro correlate of immune suppression in vivo? *Infect Immun* 1975;11:1261-4.
- Pavia CS, Folds JD, Baseman JB. Selective in vitro response of thymus-derived lymphocytes from *Treponema pallidum*-infected rabbits. *Infect Immun* 1977;18:603-11.
- Maret SM, Baseman JB, Folds JD. Cell-mediated immunity in *Treponema pallidum* infected rabbits: in vitro response of splenic and lymph node lymphocytes to mitogens and specific antigens. *Clin Exp Immunol* 1980;39:38-43.
- Metzger M, Podwinska J, Smogor W. Cell mediated immunity in experimental syphilis in rabbit. *Arch Immunol Ther Exp (Warsz)* 1977;25:25-34.
- Pedersen NS, Axelsen NH, Petersen CS. Antigenic analysis of *Treponema pallidum*: cross-reactions between individual antigens of *T pallidum* and *T Reiter*. *Scand J Immunol* 1980;13:143-50.
- Lukehart SA, Baker-Zandar SA, Sell S. Characterization of lymphocyte responsiveness in early experimental syphilis. I. In vitro response to mitogens and *Treponema pallidum* antigens. *J Immunol* 1980;124:454-60.
- Wicher V, Wicher K. In vitro cell response of *Treponema pallidum*-infected rabbits. I. Lymphocyte transformation. *Clin Exp Immunol* 1977;24:480-6.
- Wicher V, Wicher K. Cell response in rabbits infected with *T pallidum* as measured by the leucocyte migration inhibition test. *British Journal of Venereal Diseases* 1975;51:240-5.
- Wicher K, Wicher V, Nakeeb SM, Dubiski S. Studies of rabbit testes infected with *Treponema pallidum*. I. Immunopathology. *British Journal of Venereal Diseases* 1983;59:349-58.
- Hanson AW, Cannefax GR. Recovery of *Treponema* and *Borrelia* after lyophilization. *J Bacteriol* 1964;88:811.
- Sober HA, Peterson EA. Protein chromatography on ion exchange cellulose. *Fed Proc* 1958;17:1116-26.
- Avrameas S, Ternyck T. The cross-linking of proteins with glutaraldehyde and its use for the preparation of immunoabsorbents. *Immunochemistry* 1969;6:53-66.
- Wicher V, Wicher K. Studies of rabbit testes infected with *Treponema pallidum*. II. Local synthesis of antibodies. *British Journal of Venereal Diseases* 1983;59:359-63.
- Wos SM, Wicher K. Antigenic evidence for host origin of exudative fluids in lesions of *Treponema pallidum*-infected rabbits. *Infect Immun* 1985;47:228-33.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680-5.
- Towbin HT, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some application. *Proc Natl Acad Sci USA* 1979;76:4350-4.
- Baker-Zander SK, Lukehart SA. Antigenic cross-reactivity between *Treponema pallidum* and other pathogenic members of the family *Spirochaetaceae*. *Infect Immun* 1984;46:116-21.
- Hanff PA, Bishop NH, Miller JN, Lovett MA. Humoral immune response in experimental syphilis to polypeptides of *Treponema pallidum*. *J Immunol* 1983;131:1973-7.
- Norgard MV, Selland CK, Kettman R Jr, Miller JN. Sensitivity and specificity of monoclonal antibodies directed against antigenic determinants of *Treponema pallidum* Nichols in diagnosis of syphilis. *Infect Immun* 1984;20:711-7.