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Serospecificity of a Cloned Protease-Resistant *Treponema pallidum*-Specific Antigen Expressed in *Escherichia coli*

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We evaluated the serological reactivity of a protease-resistant antigen designated 4D which was encoded by *Treponema pallidum* DNA and was expressed in *Escherichia coli* from recombinant plasmid pAW329. This 19,000-molecular-weight antigen was purified in its native, non-protease-treated form from *E. coli* sonic extracts by molecular sieving and ion-exchange chromatography. Antibody binding to antigen 4D was detected by a radioimmunoassay. Antigen 4D-specific antibody was detected in 95% of the sera in a Centers for Disease Control syphilis serum panel. It was also detected in 55% of 121 primary syphilis patients, whereas syphilis antibody was detected in 83% of the sera by a fluorescent treponemal antibody absorption test and in 88% of the sera by a *T. pallidum* microhemagglutination test. In tests of 118 normal sera, less than 3% demonstrated antibody to antigen 4D; these results are similar to microhemagglutination and fluorescent treponemal antibody absorption test results. Rabbit antisera against *Treponema phagedenis*, *Treponema refringens*, *Treponema denticola*, and *Treponema vincentii* did not react with antigen 4D.

The use of recombinant DNA technology to obtain individual pathogen antigens may have important applications for the diagnosis of infectious diseases. Improved sensitivity and specificity are potential advantages which antigens encoded by cloned pathogens have over partially purified antigens for serodiagnostic infectious disease assays. These advantages could be realized by selection of highly immunogenic non-cross-reactive antigens having diagnostic significance (2). The use of antigens encoded by cloned *Treponema pallidum* DNA for a syphilis serologic test has the additional potential advantage of providing a reproducible source of treponemal antigens without the well-documented difficulties (4) of *T. pallidum* cultivation.

The cloning and expression of *T. pallidum* DNA in *Escherichia coli* have been described by several investigators (11, 12). While treponemal antigens expressed by *E. coli* may have potential use in the serodiagnosis of syphilis, complete seroreactivities of these antigens have not been extensively characterized. Therefore, we undertook further serological evaluation of a previously described protease-resistant syphilis antigen expressed in *E. coli* (3). The original clone was made from a Charon 30 library of *T. pallidum* DNA and was designated Tp4D. Plasmid subclones were produced in pBR322. Plasmid subclone pAW329 was used in this study. The treponemal specific protein previously designated antigen 4D and expressed by this plasmid in *E. coli* was purified and used as an antigen to evaluate sera from syphilis patients.

MATERIALS AND METHODS

Purification of antigen. Native non-protease-treated antigen 4D was purified as previously described (3), except that all buffers contained 5 mM dithiothreitol.

Gel electrophoresis. Discontinuous sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis was performed with stacking and separating gels containing 3 and 15% acrylamide, respectively. Samples that contained 2% sodium dodecyl sulfate, 1% dithiothreitol, and bromphenol blue as the tracking dye were added in 50-μl amounts. Electrophoresis was performed with a constant current of 24 mA. Slab gels were fixed and stained with Coomassie blue.

RIA. For the radioimmunoassay (RIA) purified native unboiled antigen was adsorbed to polyvinyl chloride microtiter plates at a concentration of 50 ng/ml in phosphate-buffered saline overnight at 4°C. The plates were then washed with phosphate-buffered saline and blocked with 0.1% ovalbumin for 30 min at RT. Patient samples were diluted in phosphate-buffered saline containing 0.1%

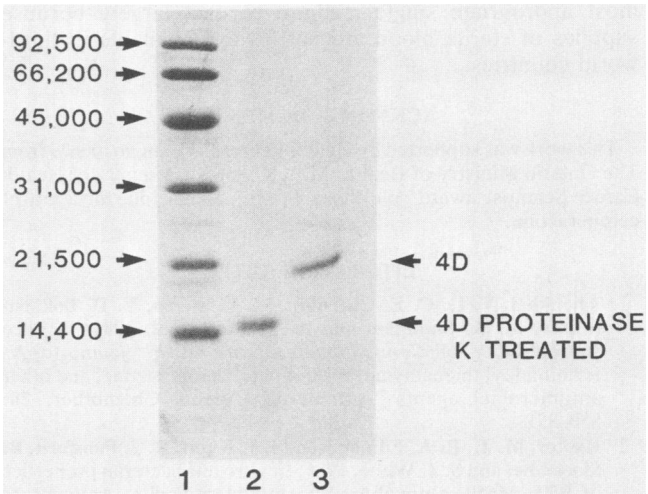

FIG. 1. Purification of antigen 4D from *E. coli* sonic extracts. Untreated purified antigen 4D and purified antigen 4D treated with protease were analyzed by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis; the samples were boiled for 2 min, and the gel was stained with Coomassie blue. Lane 1, Molecular weight markers; lane 2, protease-treated antigen; lane 3, untreated antigen.

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ovalbumin before being added to the wells and were allowed to incubate for 2 hours at RT. After the plates were washed, labeled protein A, ^{125}I-labeled goat anti-human immunoglobulin G (IgG-IgA-IgM), or goat anti-human IgM was used to detect any antibody binding to antigen 4D, as previously described (8).

Reference syphilis antibody tests. The fluorescent treponemal antibody absorption (FTA-ABS) test was performed by using a FTA-ABS Fluoro-Kit II (Clinical Sciences, Inc.) according to the directions of the manufacturer.

The microhemagglutination treponemal antibody (MHA-TP) test was performed by using the SERA-TEK treponemal antibody test (Ames Div., Miles Laboratories, Inc.) according to the directions of the manufacturer.

The rapid plasma reagin test was performed by using the Macro-Vue RPR Card Test (Becton Dickinson and Co.) according to the directions of the manufacturer.

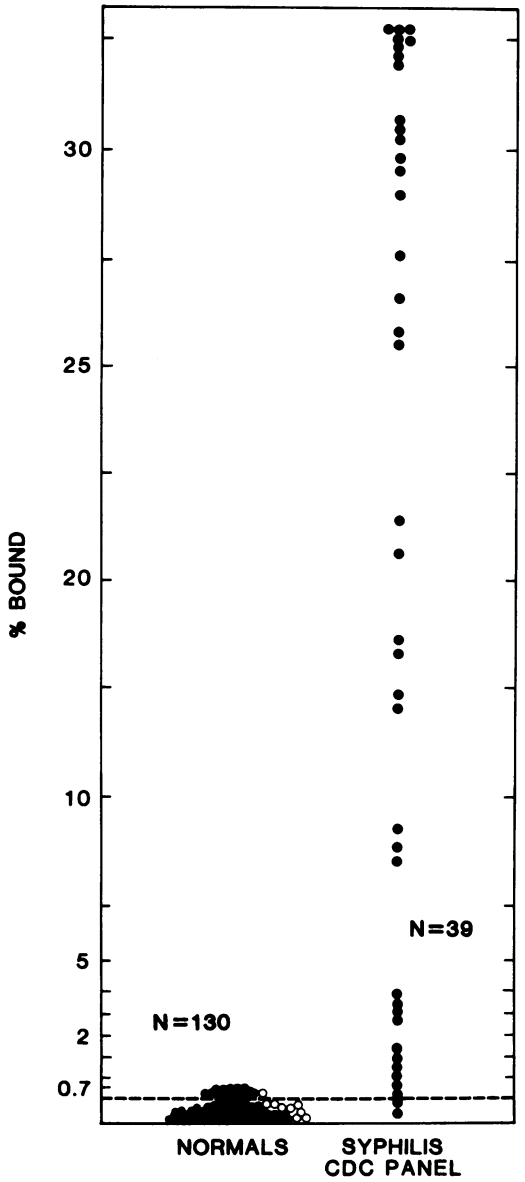

FIG. 2. Distribution of bound ^{125}I-labeled protein A detecting antigen 4D-specific antibody in normal patient sera and the CDC syphilis serum panel. The open circles represent biological false-positive patient sera.

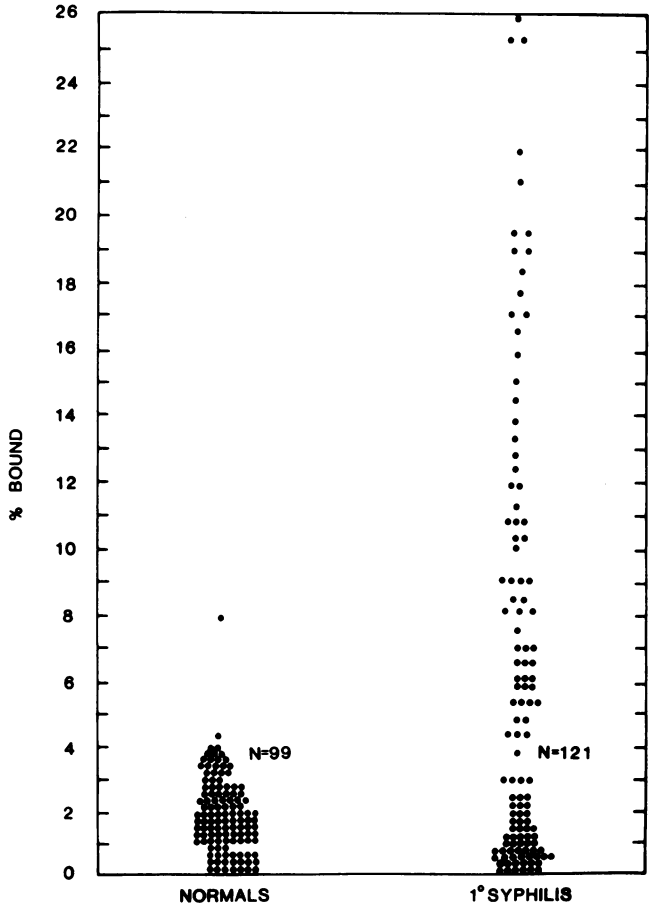

FIG. 3. Distribution of bound ^{125}I-labeled protein A detecting antigen 4D-specific antibody in normal patient sera and primary syphilis sera.

Sera. Sera were obtained from untreated primary syphilis patients. These patients had chancre-suspected lesions as diagnosed by physicians at venereal disease clinics, and were positive according to one or more of the serological tests for syphilis antibody. These syphilis sera were obtained from the San Diego Department of Health Services, the Florida Department of Health and Rehabilitation (Tampa Branch Laboratory), the New York Department of Public Health, the Pennsylvania Health Department, and the Houston Health Department. A syphilis serum panel was obtained from Sandra Larsen, Centers for Disease Control (CDC). These sera were from patients with known primary, secondary, latent, and late cases of syphilis; also included were biological false-positive sera. Rabbit antisera to *Treponema phagedenis* biotype Reiter, *Treponema refringens*, *Treponema denticola*, and *Treponema vincentii* were obtained from Elizabeth Hunter, CDC. The normal patient sera used were rapid plasma reagin test-negative sera obtained from patients during routine physical examinations.

RESULTS

Purification of antigen. As shown in Fig. 1, antigen 4D purification from *E. coli* sonic extracts resulted in a single Coomassie blue-stained band in sodium dodecyl sulfate-

TABLE 1. *T. pallidum*-specific antibody detected in primary syphilis patient sera and normal patient sera

Assay	% of positive sera		Relative sensitivity (%)[a]	Relative specificity (%)[b]
	Primary syphilis sera	Rapid plasma reagin test-negative sera		
Antigen 4D RIA	55 (67/121)[c]	2.5 (3/118)	67	97.5
FTA-ABS	83 (100/121)	2.5 (3/118)	99	97.5
MHA-TP	88 (107/121)	1.6 (2/118)	100	98.4

[a] Calculated as follows: (number of positive sera/total number of sera for which two of three tests were positive) × 100.

[b] Calculated as follows: (number of negative sera/total number of sera for which two of three tests were negative) × 100.

[c] The numbers in parentheses are number of positive sera/number of sera tested.

polyacrylamide gel electrophoresis preparations at a molecular weight of approximately 19,000. This antigen exhibited resistance to proteinase K treatment, as previously reported (3). The unboiled, non-proteinase K-treated antigen with a molecular weight of approximately 190,000 (3) was used in the RIA.

CDC syphilis serum panel. Samples from the CDC syphilis serum panel (4 untreated primary, 7 treated primary, 5 untreated secondary, 10 treated secondary, 10 treated early latent, and 3 late latent patient sera) and sera from random normal patients were tested by using the RIA and a patient serum dilution of 1:100. The distribution of bound radioactivity obtained by using purified antigen 4D and ^{125}I-labeled protein A to detect bound antibody is shown in Fig. 2. When we used a cutoff point of the mean plus two standard deviations of the mean of the negative samples, 37 of 39 samples were considered positive. Of the two samples considered negative, one was from a primary patient, and the other was from a late latent patient. Of the 130 negative sera, 117 were below this cutoff point, including 7 of 8 biological false-positive specimens.

Primary syphilis sera. Sera from 121 untreated primary syphilis patients and sera from 130 negative patients were tested by using the RIA and patient serum dilution of 1:25. These sera were also tested by using a reference MHA-TP test and an FTA-ABS test. The distribution of bound radioactivity obtained by using purified antigen 4D and ^{125}I-labeled protein A to detect antibody is shown in Fig. 3. When we used a cutoff point of the mean plus two standard deviations of the mean of negative samples tested, 55% of the samples were positive as determined by the antigen 4D RIA. As determined by the FTA-ABS test, 83% of the primary samples were positive, and as determined by the MHA-TP test 88% were positive (Table 1).

Testing of antigen 4D antibody-negative primary syphilis sera. Because protein A does not detect IgM and detects only some IgG isotypes, the use of a polyvalent anti-human IgG-IgM-IgA probe and an anti-human IgM probe in the RIA was evaluated. This was accomplished by testing all primary syphilis sera in which no antigen 4D-specific antibody was detected with the protein A probe. The distribution of bound radioactivity in the tests of these sera is shown in Fig. 4. Again, we used the mean plus two standard deviations of the mean of 53 normal serum samples that were also tested with these probes for the cutoff point. As shown in Table 2, the polyvalent probe identified three additional primary syphilis serum specimens as positive. The anti-IgM probe did not appear to identify any additional primary sera as positive.

Reactivity with nonpathogenic treponemes. Antisera from the CDC prepared in hyperimmunized rabbits against various nonpathogenic treponemes were assayed for cross-reactivity antibody to antigen 4D (Table 3). No significant cross-reactive antibody to antigen 4D was detected with antisera against *T. phagedenis* biotype Reiter, *T. denticola*, *T. vincenti*, and *T. refringens*.

DISCUSSION

The ability of cloned *T. pallidum* specific antigen 4D to detect syphilis antibody was demonstrated by the results obtained by testing the CDC syphilis patient serum panel.

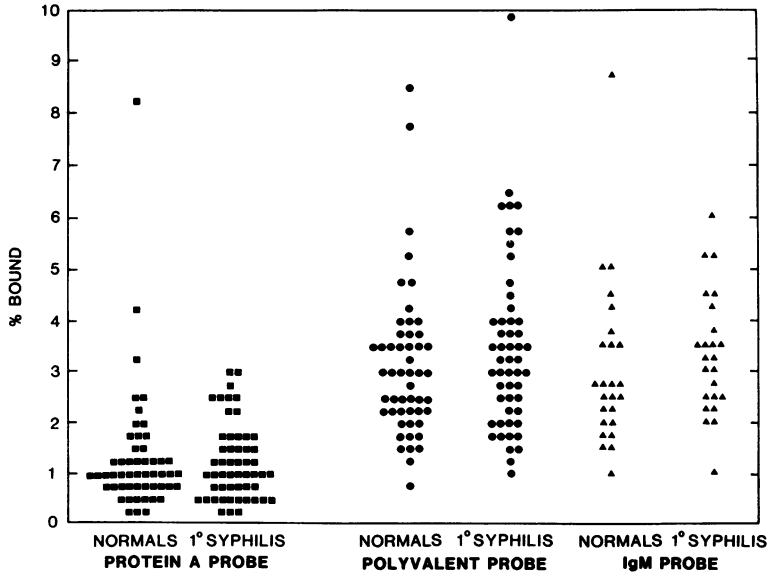

FIG. 4. Distribution of antigen 4D-specific antibody which detected bound ^{125}I-labeled probes (anti-human IgG-IgM-IgA antibody, anti-human IgM antibody, and protein A) in normal and primary syphilis sera. The primary syphilis sera selected for testing were negative for protein A-detected antigen 4D-specific antibody, as determined by prior testing.

Antibody to antigen 4D was detected in the sera of 95% of the patients. This observation confirms the results obtained previously by Fehniger et al. (3) and by us (W. Buhles, M. Lovett, H. Erlich, G. Rodgers, and P. Vaillancourt, Program Abstr. 23rd Intersc. Conf. Antimicrob. Agents Chemother. abstr. no. 670, 1983) in assays of antibody to this antigen by immunoblotting. In addition, no antibody specific for antigen 4D was detected in more than 97% of the normal sera or biological false-positive sera tested.

The use of syphilis-specific cloned antigens in syphilis serological tests has the potential advantage of eliminating the use of sorbate. Sorbate currently must be used with treponemal antibody tests to eliminate false-positive reactions, apparently due to the presence of antibody to *T. phagendenis* biotype Reiter or other nonpathogenic treponemes (7). In our limited testing, no cross-reactivity was observed with nonpathogenic treponemes and antigen 4D.

A potential problem associated with the use of a recombinant syphilis antigen in a serological assay is the possibility of *E. coli* antigen contamination. Any *E. coli* contaminants contained in the antigen preparation could cause false-positive reactions, since antibody to *E. coli* would also be detected. We observed *E. coli*-specific antibodies to a variety of *E. coli* antigens in the majority of patients assayed by immunoblotting when partially purified antigen 4D was used (unpublished data). The absence of antigen 4D-specific antibody in the large panel of normal sera tested indicates that little, if any, antibody to *E. coli* is detected by using this antigen 4D preparation.

The accurate serodiagnosis of primary syphilis is of critical importance for any syphilis serological test. Differences in the ability of various syphilis serological tests to detect antibody in primary disease have been described previously (1, 6). Consequently, serum samples from patients in this stage of disease were chosen for an expanded study with antigen 4D. The results obtained in our RIA with purified antigen 4D indicated that detectable IgG or IgM antibody to antigen 4D was absent in approximately one-third of primary syphilis patient sera in which syphilis antibody was detectable by the MHA-TP and FTA-ABS assays (Fig. 3).

The inability to detect an antibody to antigen 4D in some of the primary patients tested may have been due to the sensitivity of the antibody assay used. Up to 67 antigenic polypeptides have been identified (10) in *T. pallidum*. The level of antibody to any one of these antigens may be difficult to detect in the early stages of the disease. Alternatively, the immune responses to certain antigens may be delayed relative to others. In primary syphilis, the immune response to many antigens may not be observed until the onset of secondary syphilis (5, 9).

Further evaluations with additional syphilis patient sera of antigen 4D and other syphilis-specific antigens encoded by cloned *T. pallidum* DNA are in progress to determine the complete diagnostic potential of antigen 4D and other treponemal antigens.

TABLE 2. *T. pallidum*-specific antibody detected in tested antigen 4D antibody-negative primary syphilis sera

Antibody assay	% of positive sera	
	Antigen 4D-specific antibody-negative primary syphilis sera[a]	Normal sera
Antigen 4D RIA with polyvalent anti-IgG-IgM-IgA probe[b]	5.6 (3/53)[c]	5.7 (3/53)
Antigen 4D RIA with anti-IgM probe[d]	0 (0/25)	4.0 (1/25)
FTA-ABS	62 (33/53)	1.9 (1/53)
MHA-TP	73.6 (39/53)	1.9 (1/53)

[a] Primary syphilis patient sera which did not contain antigen 4D-specific antibody, as determined by using radioactively labeled protein A.

[b] Sera were assayed at a dilution of 1:50 to antigen 4D absorbed to polyvinyl chloride 96-well plates. Bound antibody was detected with ^{125}I-labeled goat anti-human IgG-IgM-IgA antibody.

[c] The numbers in parentheses are number of positive sera/number of sera tested.

[d] Sera were assayed at a dilution of 1:50 to antigen 4D absorbed to polyvinyl chloride 96-well plates. Bound IgM antibody was detected with ^{125}I-labeled goat anti-human IgM antibody.

TABLE 3. Reactivity of antigen 4D with rabbit antisera to nonpathogenic treponemes

Rabbit antiserum	% of antibody bound to antigen 4D[a]	% of antibody bound to *T. phagedenis* antigen[b]
Anti-*T. phagedenis* from rabbit 2497	1.0	34.0
Prebleed from rabbit 2497	0.5	1.0
Anti-*T. phagedenis* from rabbit 2498	0.5	30.0
Anti-*T. denticola*	0.7	ND[c]
Anti-*T. vincentii*	0.3	ND
Anti-*T. refringens*	2.0	ND
Anti-4D	50.0	ND

[a] Rabbit antisera were assayed at a dilution of 1:50 to antigen 4D absorbed to polyvinyl chloride 96-well plates. Bound antibody was detected with ^{125}I-labeled protein A.

[b] Rabbit antisera were assayed at a dilution of 1:50 to *T. phagedenis* biotype Reiter sonic extract absorbed to polyvinyl chloride 96 well-plates. Bound antibody was detected with ^{125}I-labeled protein A.

[c] ND, Not done.

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