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Molecular Basis of Immunological Cross-Reactivity Between *Treponema pallidum* and *Treponema pertenue*

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Protein antigens of *Treponema pallidum*, Nichols strain, and *Treponema pertenue*, Gauthier strain, were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting techniques. Treponemal proteins were solubilized in 1% sodium dodecyl sulfate, electrophoresed on 12.5% polyacrylamide gels, and either stained with Coomassie brilliant blue or electrophoretically transferred to nitrocellulose paper. These antigen blots were incubated with sera from rabbits infected with either *T. pallidum* or *T. pertenue* and ^{125}I-labeled staphylococcal protein A and exposed to X-ray film for visualization of antigenic molecules. Protein profiles of each organism separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue showed no distinguishable differences. Antigenic profiles as determined by Western blots were similar with two exceptions. A 39,500-dalton band was present on *T. pertenue* but absent from *T. pallidum*, and a 19,000-dalton band was present on *T. pallidum* but absent from *T. pertenue* (although two additional antigenic bands at 21,000 and 18,000 daltons were seen on *T. pertenue*). Because these differences were detected by using antisera raised against either *T. pallidum* or *T. pertenue*, these molecules must contain some antigenic determinants in common despite their differences in molecular weight.

Members of the genus *Treponema* cause a group of chronic granulomatous diseases with significant worldwide prevalence. This genus includes the human pathogens *T. pallidum* (venereal and endemic syphilis), *T. pertenue* (yaws), and *T. carateum* (pinta). The organisms are unicellular bacteria which exhibit a characteristic spiral shape and corkscrew motility (15). Fieldsteel et al. have achieved replication of *T. pallidum* in vitro (3), but continuous cultivation of these pathogens outside the human host or appropriate animal model has met with failure (6). These organisms are morphologically indistinguishable and cannot be identified as to species on the basis of conventional serological tests for syphilis (19). Rather, they have been classified according to the disease which each produces.

Immunological cross-reactivity has previously been reported among the *Treponema* spp.; several challenge experiments have demonstrated variable degrees of cross-immunity. Rabbits infected with *T. pallidum*, Nichols strain, and subsequently challenged with *T. pertenue* showed various degrees of protection against symptomatic infection, depending on the strain of *T. pertenue* used (20). The gamma-irradiated vaccine described by Miller, although providing complete protection to challenge with homologous *T. pallidum*, Nichols strain, provided only minimal resistance to challenge with *T. pertenue* (13). A study designed to test immunity to asymptomatic infection by treating syphilis- or yaws-infected rabbits with penicillin showed that 50% of animals infected with *T. pallidum* and challenged with *T. pertenue* were fully protected, whereas only 25% of rabbits infected with *T. pertenue* and challenged with *T. pallidum* were protected (20). Hamsters infected with *T. pallidum*, Nichols strain, or *T. pertenue* also developed complete resistance to symptomatic infection when challenged with heterologous organisms (14).

Cross-immunity is also confirmed by serological tests. Khan et al. demonstrated cross-reacting, immobilizing antibodies between *T. pallidum* and *T. pertenue* (7). Cross-agglutination titers and *T. pallidum* immobilization titers yielded similar results (20). Finally, immunofluorescence studies of paraffin-embedded sections of lymph nodes from hamsters infected with *T. pertenue* or *T. pallidum* demonstrated that the treponemes stained equally well in the heterologous system (1).

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The present investigation was undertaken to examine more closely the nature of immunological cross-reactivity between *T. pallidum* and *T. pertenue*. Common protein antigens on *T. pallidum*, Nichols strain, or *T.* pertenue, Gauthier strain, were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting techniques by using antisera produced against each organism.

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MATERIALS AND METHODS

Animals. Adult male New Zealand white rabbits were obtained from a local supplier (R & R Rabbitry, Stanwood, Wash.). Each rabbit was tested for evidence of *T. paraluis-cuniculi* infection by the Venereal Disease Research Laboratory (VDRL) and the fluorescent treponemal antibody-absorbed (FTA-ABS) tests. Only those rabbits with nonreactive serological tests and no clinical signs of infection were included in this study. All rabbits were housed individually at 19 to 20°C and were given antibiotic-free food and water.

Source of treponemes. *T. pallidum*, Nichols strain, was propagated in rabbits by testicular passage as previously described (11). *T. pertenue*, Gauthier strain, was obtained from the Centers for Disease Control, Atlanta, Ga., and was propagated in rabbits by monthly testicular passage.

Sera and antisera. Nonimmune (normal) rabbit serum was obtained from four VDRL- and FTA-ABS-nonreactive rabbits and pooled. A pool of rabbit anti-*T. pallidum* antiserum was obtained from four rabbits 3 to 4 months after intratesticular infection. A pool of rabbit anti-*T. pertenue* antiserum was collected from four rabbits 10 days to 3 months after intratesticular infection. All antisera were collected by cardiac puncture or from the medial ear artery and allowed to clot at room temperature for 2 h in sterile glass tubes. Clotted blood was centrifuged 250 × *g* for 10 min; the serum was withdrawn and samples were stored frozen at −20°C.

Serological tests. The VDRL slide flocculation and FTA-ABS tests were performed as described previously (2) with modifications (10). Antisera were tested for reactivity against *T. pallidum* and *T. pertenue* by an immunofluorescence test. Approximately 2×10^5 washed organisms were placed on slides, allowed to air dry, and fixed with 10% methanol for 20 s. Serial twofold dilutions of antisera were made in phosphate-buffered saline and tested as in the FTA test (2) by using fluorescein isothiocyanate-conjugated goat anti-rabbit antiserum (1:1,600; Cappel Laboratories, Cochranville, Pa.). Slides were examined on a Zeiss fluorescence microscope, and the degree of fluorescence was recorded on a 1 to 4^+ scale.

SDS-PAGE. *T. pallidum* antigens were prepared by the method of Lukehart et al. (9). *T. pertenue* antigens were similarly prepared except that the rabbits were given cortisone twice weekly and sacrificed 4 weeks postinfection. Treponemal proteins were electrophoresed on 12.5% polyacrylamide slab gels in the discontinuous Tris-glycine system as described by Laemmli (8). Gels were fixed and stained with 0.25% Coomassie brilliant blue or were used for Western blotting. Protein standards for estimating molecular weights (Bio-Rad Laboratories, Richmond, Calif.) were included on each gel. Approximately molecular weights were determined by the method of Weber and Osborn (22).

Western blots. Electrophoretic transfer of proteins to nitrocellulose paper (Western blot) was performed by the technique of Towbin et al. (16) with a Trans Blot Cell (Bio-Rad). Proteins were transferred at 5 to 7 volts for 18 h at room temperature. Staining of Western blots was performed as previously reported (9) except that antisera (1:100) and ^{125}I-labeled staphylococcal protein A (New England Nuclear Corp., Boston, Mass.) were diluted in 0.5% bovine serum albumin–10 mM Tris-saline, pH 7.4, containing 0.2% Triton X-100.

RESULTS

Serological reactivity of antisera used for Western blotting. Antisera prepared against *T. pallidum* and *T. pertenue* were tested undiluted and in twofold serial dilutions by immunofluorescence against the homologous and heterologous organisms. In each case, the undiluted antiserum reacted with 4^+ fluorescence. *T. pallidum* antiserum had a titer of 1:1,024 against both organisms. *T. pertenue* antiserum had a titer of 1:1,024 against *T. pallidum* and 1:512 against *T. pertenue*. The endpoint of the test was the last dilution to yield 2^+ fluorescence.

Protein profiles of *T. pallidum* and *T. pertenue*. *T. pallidum*, Nichols strain, and *T. pertenue*, Gauthier strain, were extracted from rabbit testes and washed to remove loosely bound host proteins, solubilized in SDS, separated by SDS-PAGE, and stained with Coomassie brilliant blue. Equal numbers of organisms were loaded in each lane. The resulting profiles are shown in Fig. 1. These profiles are essentially identical and are comprised of at least 35 distinct protein-containing bands. The intensities of some bands differ between the two organisms, indicating that differing quantities of these proteins might be represented in each organism.

Antigens of *T. pallidum* and *T. pertenue*. Common antigens of *T. pallidum* and *T. pertenue* were identified by SDS-PAGE and Western blotting techniques. Treponemal antigens were solubilized in 1% SDS, electrophoresed on 12.5% polyacrylamide gels, and electrophoretically transferred to nitrocellulose paper. These blots were incubated with antiserum prepared against either *T. pallidum* or *T. pertenue* and were then incubated with ^{125}I-labeled staphylococcal protein A. The resulting antigens were visualized by exposure to X-ray film (Fig. 2).

When the antigenic profiles of *T. pallidum* (lanes A) and *T. pertenue* (lanes B) are compared, common antigenic molecules are identi-

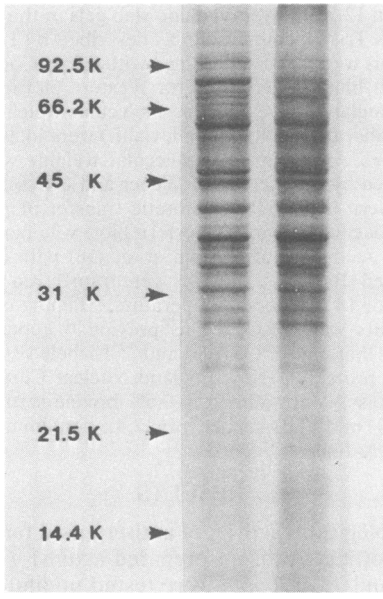

FIG. 1. Protein profiles of SDS-solubilized *T. pallidum*, Nichols strain (left), and *T. pertenue*, Gauthier strain (right), after 12.5% SDS-PAGE and staining with Coomassie brilliant blue. Equal numbers of organisms were loaded on each lane. No obvious differences in the positions of bands are evident. Molecular weight markers are shown at the left.

fied, with apparent molecular weights of 69,000; a broad band at 48,000; a band of 41,000; a triplet of 37,000, 35,000, and 33,000; a single band at 30,000 and 28,000; and a doublet at 14,000 and 12,000.

Several molecules, however, are identified which have no direct correlate in the other organism. A band with an apparent molecular weight of 39,500 is seen on *T. pertenue* but is absent from *T. pallidum*. *T. pallidum* contains an antigenic molecule at 19,000 daltons, and although no band is found in that position in *T. pertenue*, two additional bands (21,000 and 18,000 daltons) are seen on *T. pertenue*. These molecular weight differences are seen with antiserum prepared against either *T. pallidum* or *T. pertenue*. Normal rabbit serum failed to react with any molecules (data not shown).

DISCUSSION

The molecular basis for antigenic cross-reactivity between *T. pallidum* and *T. pertenue* was examined by using the techniques of SDS-PAGE and Western blotting. We have shown that the molecular differences between these two closely related human pathogens are extremely subtle. Antigenic molecules of *T. pallidum* revealed by antiserum raised against either *T. pallidum* or *T. pertenue* and ^{125}I-labeled staphylococcal protein A showed no differences in profiles. Likewise, blots of *T. pertenue* stained with each antiserum were identical. However, when the profiles of *T. pallidum* and *T. pertenue*, stained with either antiserum, were compared, two major differences were detected. An antigenic band having an apparent molecular weight of 39,500 was present on *T. pertenue* but absent from *T. pallidum*, and a band having an apparent molecular weight of 19,000 was present on *T. pallidum* but absent from *T. pertenue*. *T. pertenue* contained two additional antigenic bands bracketing either side of this position. Inasmuch as these differences may be detected with antiserum raised against either species, this may be indicative of common antigenic determinants residing on peptides with different molecular weights. Such a phenomenon might indicate alterations in amino acid sequence or differing degrees of glycosylation of putative cell coat glycoproteins.

Although Western blotting techniques allow the examination of antigenic molecules to be performed despite the difficulties of obtaining large numbers of purified pathogenic treponemes, there are limitations to the technique. Because SDS was used to solubilize treponemes, substantial secondary, tertiary, and quaternary structure may subsequently be lost, pre-

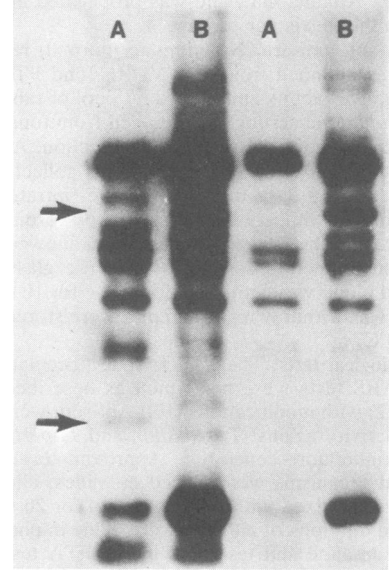

FIG. 2. Antigens of *T. pallidum*, Nichols strain (A), and *T. pertenue*, Gauthier strain (B), identified by staining with pooled rabbit anti-*T. pallidum* antiserum (lanes 1 and 2) or pooled rabbit anti-*T. pertenue* antiserum (lanes 3 and 4) and ^{125}I-labeled staphylococcal protein A (Western blot technique). The arrows indicate molecular weight differences of bands with common antigens between lanes A and B on either set of profiles.

venting the examination of antigens with differences in conformational determinants. Therefore, only SDS-stable determinants can be studied by this approach. Antigens which fail to migrate and bind to nitrocellulose would also be overlooked. Further, with this technique the existence of one or more common antigenic determinants on a given molecule would mask differences at other sites on the same molecule. Thus, minor, though perhaps important, antigenic differences would not be detected here. These differences could explain the lack of complete protection in previously performed challenge experiments, as well as variations in serological reactivity between *T. pallidum* and *T. pertenue*. Currently, *T. pertenue* antiserum fully absorbed with *T. pallidum*, Nichols strain, is being prepared in an attempt to determine which molecules contain antigenic determinants unique to *T. pertenue*. Monoclonal antibodies produced against *T. pallidum* are also being tested for their reactivity against *T. pertenue* in Western blots. The identification of such species-specific antigens could provide useful tools in the differential serodiagnosis of syphilis and yaws.

The titers of the pooled rabbit antisera used in this study were essentially equivalent as determined by immunofluorescence on both homologous and heterologous treponemes. These results suggest the presence of substantial numbers of common antigens as subsequently seen by Western blotting. It is evident that immunofluorescence testing with polyclonal antibodies lacks the sensitivity necessary to distinguish differences between such closely related organisms.

Within the genus *Treponema*, previous immunological comparisons have been limited to studies of immunity to rechallenge with different species and to serological cross-reactivity among the species; specific molecular variations have not previously been described. Challenge studies in rabbits have shown that initial infection with *T. pallidum* or *T. pertenue* affords some reciprocal immunity upon challenge with substantial numbers of heterologous treponemes (21). This protection is seen as a delay in the onset of lesion development. Cross-protection to challenge with *T. pallidum*, Nichols strain, or *T. pertenue* can also be shown in the hamster model of endemic syphilis (14). However, this model does not yield definitive data with regard to cross-protection studies because symptomatic infection with *T. pallidum* Nichols strain, does not regularly occur in hamsters. In humans, evidence exists for substantial cross-protection; 0 of 10 latent syphilitics inoculated with *T. pertenue* developed lesions (18). Turner (18) also stated that clinically recognizable syphilis rarely occurs among persons giving a history of yaws or among persons living in a yaws-endemic region.

Differences between *T. pallidum* and *T. pertenue* based on serological reactivity are even harder to distinguish. No differences in the reactions of 12 yaws and 12 syphilitic sera were detected when either *T. pallidum* or *T. pertenue* was used as an antigen in the FTA-ABS or *T. pallidum* immobilization test (5). However, Khan et al. (7) showed that sera tested in the *T. pallidum* immobilization test had, in general, 10-fold-greater immobilization capacity against homologous treponemes as compared with heterologous treponemes. Immunofluorescent staining of lymph nodes from hamsters infected with *T. pallidum* or *T. pertenue* showed that treponemes stained equally well when the heterologous antiserum was used (1).

Studies of treponemal attachment to cultured mammalian cells also revealed differences between *T. pallidum* and *T. pertenue*. *T. pertenue* attached in fewer numbers and survived for a shorter period of time than *T. pallidum* (4).

These differences may in one sense be considered minor since DNA sequence homology studies have shown that *T. pallidum* and *T. pertenue* are identical within the limits of resolution of that technique (2% of the entire genome, or ca. 180 peptides) (12). However, if those results are accurate, then those few peptides may be of considerable importance inasmuch as these separate species continue to consistently produce different clinical syndromes both in humans (17) and in rabbits (18). The incomplete nature of protection between species of the genus *Treponema* indicates that the unique antigenic epitopes are probably very important in conferring immunity and might therefore be considered possible candidates for vaccines.

Studies of the antigenic structures of the pathogenic treponemes should eventually result in the dissection of their antigenic profiles into genus and species-specific antigens. Such information should prove useful for both the development of general or specific vaccines and improved serodiagnostic techniques. Such studies may also yield clues regarding the evolution and natural history of the various pathogenic treponemes.

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