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Specificity of Antibodies from Patients with Pinta for Antigens of *Treponema pallidum* Subspecies *pallidum*

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Inhabitants of a remote Panamanian village were examined for clinical and serological evidence of pinta infection. Of 104 persons examined, 21 (20%) had clinical evidence of active or inactive pinta, and 54 (52%) were seropositive. Sera were evaluated for antibody to individual *Treponema pallidum* antigens. Sera from all four patients with active pinta contained antibody to the 47–48-kilodalton major antigen; the intensity of reactivity and the number of antigens recognized increased with age and, presumably, duration of infection. Sera from six children with inactive pinta reacted strongly with multiple *T. pallidum* antigens, whereas adults with inactive pinta had less intense reactivity against fewer molecules. Seronegative controls demonstrated only weak reactivity to fewer than five molecules. The development of antibody reactivity to the full spectrum of *T. pallidum* antigens during the course of infection demonstrates the high degree of antigenic relatedness of *T. pallidum* and *Treponema carateum* and is similar to the development of humoral responsiveness during syphilis infection.

The pathogenic treponemes cause chronic granulomatous diseases (venereal syphilis, endemic syphilis, yaws, and pinta) affecting both adults and children worldwide. These organisms are morphologically and serologically indistinguishable; therefore, speciation has been based primarily on clinical and epidemiological considerations. The causative agents of venereal and endemic syphilis and yaws have been successfully passaged in rabbits and hamsters, a process providing both a source of organisms for study and an experimental model. On the basis of DNA homology studies [1, 2], these organisms have recently been reclassified as subspecies of *Treponema pallidum*. Despite limited reports of experimental infection in the chimpanzee [3, 4], no animal model has been developed for *Treponema carateum* propagation; consequently, *T. carateum* is the least studied of the pathogenic treponemes and is still considered to be a separate species.

Pinta is the most benign of the treponematoses [5–7]; the disease causes only discoloration of the skin as a long-term sequela. It has been hypothesized that pinta is the first of the treponemal diseases and may be the disease from which yaws, endemic syphilis, and venereal syphilis evolved [5, 6]. Infection occurs through skin or mucous membrane contact before adolescence and is manifested as early-active disease, followed by latent (inactive) infection, a course characteristic of the treponematoses. Primary lesions are usually observed among infants and children ≤15 y of age. Late lesions ("pintids") affect adults in the population. Pinta is confined to Central and South America, with most cases reported in Colombia and Mexico [5, 7]. Before 1950, there were an estimated one million cases [7]; subsequent mass treatment campaigns and improved hygiene have dramatically reduced the incidence of the infection, although no accurate estimate of its current prevalence is available.

Antigenic cross-reactivity has been observed among the pathogenic treponemes; infection with any of them will induce reactivity in both nontreponemal and treponemal tests for syphilis [5–8]. Cross-agglutinating and cross-immobilizing antibodies and limited cross-protection between the pathogenic treponemes have also been observed [8–13]. Very little is known of the antigenic structure of

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T. carateum, although recent progress has been made by using antisera from humans [14–18] or rabbits [19–24] with syphilis or yaws infection to identify at least 22 *T. pallidum* antigens. This paper describes the spectrum of antibody reactivity against individual molecules of *T. pallidum* ssp. *pallidum* in patients with active and inactive pinta.

Subjects and Methods

Subjects. During annual visits by a medical team in 1982 and 1983, serum specimens were collected from 104 of 205 inhabitants of a remote Panamanian village (located 90 miles east of Panama City) in which pinta had previously been described. Mass treatment reportedly took place in both the 1960s and 1970s; however, accurate information regarding treatment of individuals in this village was not available. Sera were stored at −20 C until use.

Serological testing. The following tests were performed as described by the Centers for Disease Control [25]: the rapid plasma reagin (RPR), the VDRL, and the fluorescent treponemal antibody absorption (FTA-ABS) tests. Titers of fluorescent IgG and IgM from selected sera were determined by modifying the standard FTA-ABS test as previously described [18]. Briefly, sera were diluted 1:5 in sorbent, and serial twofold dilutions were made in PBS. FITC-conjugated goat antibody to human IgG (heavy-chain specific) and to human IgM (heavy-chain specific; Cappel Laboratories, Malvern, Pa) were used as the second antibodies at previously determined working titers of 1:1200 and 1:2000, respectively. The degree of fluorescence was recorded on a scale of 1 to 4+, and the titer was determined as the highest dilution that yielded 2+ fluorescence.

Source of organisms. *T. pallidum* ssp. *pallidum*, Nichols strain, was propagated in adult, male New Zealand white rabbits (R and R Rabbitry, Stanwood, Wash) as previously described [26]. Before use, all rabbits were tested for evidence of infection with *Treponema paraluiscuniculi* by the RPR, VDRL, and FTA-ABS tests. Rabbits were housed individually at 19–20 C and given antibiotic-free food and water.

Preparation of antigens and SDS-PAGE. *T. pallidum* antigens were prepared by the method of Lukehart et al. [27]. Whole, washed treponemes were solubilized in 1% SDS and electrophoresed on 12.5% polyacrylamide slab gels in the discontinuous Tris-glycine system, as described by Laemmli [28], by using a Hoefer SE-600 apparatus (Hoefer Scientific Instruments, San Francisco). Gels were 1.5 mm thick and 12 cm long. Approximately 2×10^8 organisms (~25 μg of protein) were loaded onto each 5-mm lane, and electrophoresis was continued until the dye front was within 1 cm of the bottom of the gel. Protein standards (97.4–12.4 kilodaltons [kDa]; Sigma, St. Louis) for estimating molecular weights were included. Approximate molecular weights were determined by the method of Weber and Osborn [29].

Immunoblotting. Electrophoretic transfer of proteins to nitrocellulose paper (0.45 μm; Schleicher and Schuell, Keene, NH) was performed by the method of Towbin et al. [30] by using a Transphor® cell (Hoefer). Proteins were transferred at 10 volts for 16–18 h at 4 C; the efficacy of transfer was determined by staining with 0.1% amido black [30]. Nitrocellulose paper was incubated with patients' sera (diluted 1:100) as previously described [18], and bound IgG was detected with ^{125}I-labeled staphylococcal protein A (New England Nuclear, Boston) at a final concentration of $2.5–5.0 \times 10^5$ cpm/mL. Sera were examined for IgM antibody, as previously described [18], by using ^{125}I-labeled goat antibody to human IgM (heavy-chain specific; Cappel Laboratories). After incubation, immunoblots were exposed to Cronex MRF 34 film (Dupont, Wilmington, Del) at −70 C with enhancing screens for 17–19 h.

The degree of antibody reactivity in the immunoblots was scored subjectively (0, 1+, 2+, 3+, 4+) on the basis of the staining intensity of protein bands and the number of antigenic molecules stained. As a guideline, reactivity of normal human sera was considered to be 0 or 1+, whereas patients with antibody reactivity that was greater than control sera were scored 2+, 3+, or 4+, depending on the range of reactivity and the staining intensity. For example, immunoblots shown in figure 3 demonstrate the following range of reactivities (left to right): 0, 1+, 2+, and 3+. Figure 4, left lane, demonstrates a 4+ reactivity score.

Results

Subjects. Of 104 village inhabitants examined, 21 had clinical evidence of active or inactive pinta. Four patients, one to eight years of age, had clinically apparent active pinta (defined as slightly raised, violaceous skin lesions) and frequently displayed a fine scale on the skin surface (figure 1, top); the remaining 17 patients, 10–91 y of age, had evidence of inactive pinta (defined as the presence of large

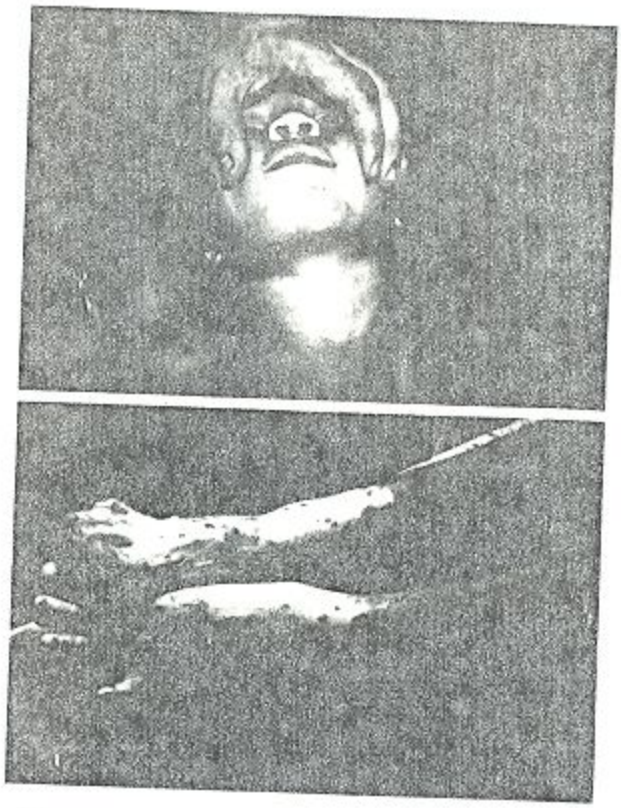

Figure 1. *Top*, active pinta lesion on the chin of a child; *bottom*, inactive, dischromic lesions on the feet of an adult.

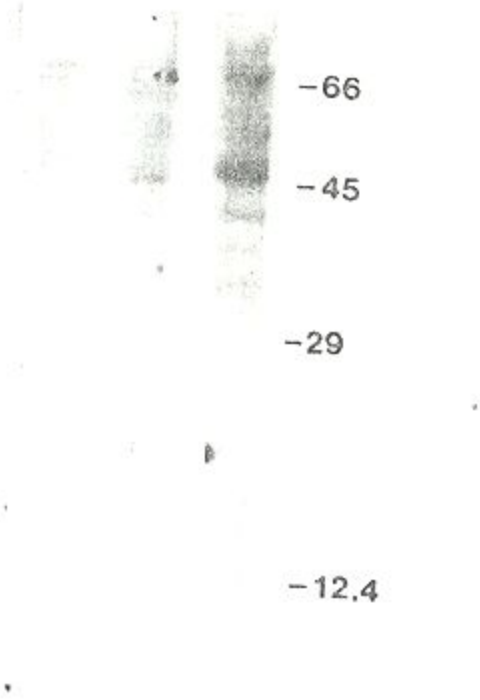

Figure 2. Antibody reactivity to antigens of *T. pallidum* in control sera from seronegative village inhabitants. Approximate molecular masses (kDa) are indicated at right.

amelanotic patches on otherwise normal-looking skin; figure 1, bottom).

Sera from 54 (52%) of the 104 subjects were reactive in the RPR test, including all 21 patients with clinical evidence of pinta. These 21 sera plus six RPR-nonreactive sera (selected as village controls) were then examined for reactivity in the VDRL and FTA-ABS tests. All evaluable patients with clinical manifestations of active or inactive pinta were reactive in both serological tests; uninfected control subjects were nonreactive. Because pinta is usually acquired before adulthood [5–7], the patients with inactive disease were separated into two age-groups for further analysis—children (10–14 y of age) and adults (19–91 y of age). The VDRL titers were not significantly different between patients with active infection (geometric mean, 11.3; range, 8–16) and children (geometric mean, 5.7; range, 1–16) or adults (geometric mean, 5.8; range, 2–16) with inactive disease ($P > .05$, Wilcoxon rank sum test).

Immunoblots. Sera from six serologically nonreactive inhabitants between the ages of seven and 19 were examined by immunoblotting with *T. pallidum* antigens; representative immunoblots are shown in figure 2. None of the control sera exhibited ≥2+ IgG reactivity to treponemal antigens. Some sera did react weakly with *T. pallidum* molecules of 47–48, 37, 34.5, and 33 kDa, as previously demonstrated by several laboratories [15–18]. These molecules contain antigenic determinants common to the nonpathogenic treponemes [27, 31], with resultant cross-reactive antibody formation.

Sera from four patients with clinical evidence of active pinta were examined (figure 3). Immunofluorescence titers of IgG antibody and patients' ages are indicated. Two (50%) of the four sera had ≥2+ reactivity with antigens of *T. pallidum*; major reactivity was directed against molecules of 47–48, 37, 14, and 12 kDa. Both immunoblot IgG reactivity and immunofluorescence titer generally increased with age.

Sera from all six children (100%) with inactive disease had ≥2+ reactivity in immunoblots; the full spectrum of *T. pallidum* antigenic molecules between 66 and 12 kDa was usually identified. Major reactivity was seen with the 47–48-, 37-, 34.5-, 33-, 30-, 14-, and 12-kDa proteins. In contrast, sera from only seven (64%) of the 11 adults with inactive pinta had ≥2+ IgG reactivity to *T. pallidum* antigens in immunoblotting. The intensity of reactivity in this group was generally lower, and the full spectrum of

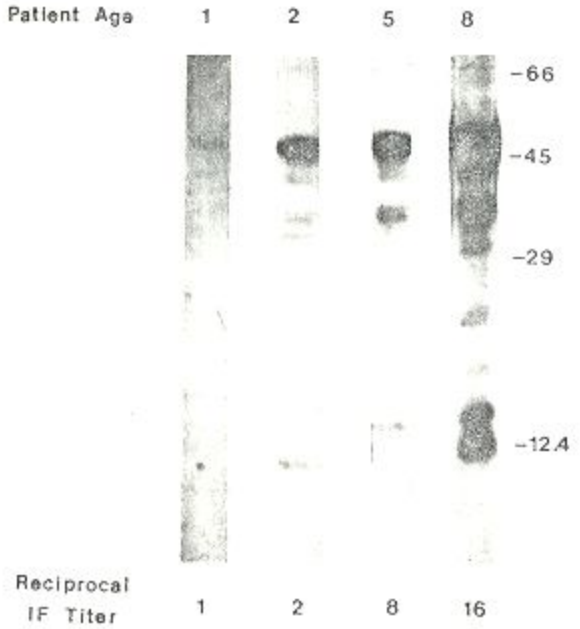

Figure 3. Antibody reactivity to antigens of *T. pallidum* in sera from patients with clinically confirmed, active pinta. Patients' ages and reciprocal IgG immunofluorescence (*IF*) titers are given. Approximate molecular masses (kDa) are indicated at right.

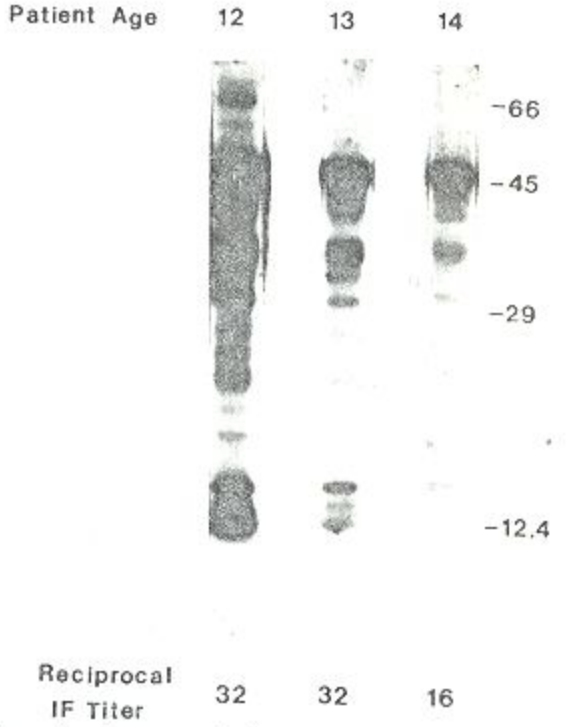

Figure 4. Representative immunoblots of sera from children (10–14 y of age) with inactive pinta. Patients' ages and the reciprocal IgG immunofluorescence (*IF*) titers are given. Approximate molecular masses (kDa) are indicated at right.

antigens was not usually recognized. Representative immunoblots of children and adults with inactive pinta are shown in figures 4 and 5, respectively.

Patients' sera and the six control specimens were tested for IgM reactivity to *T. pallidum* antigens in immunoblots. Weak reactivity was seen to the 66-, 47–48-, and 37-kDa molecules. Sera with IgM reactivity were present in some patients from all groups, and no consistent pattern was observed. Immunoblots of control sera failed to show IgM reactivity. Specific IgM reactivity in the FTA-ABS test was present in only three of the patients with inactive pinta, of whom two were children (both, 10 y of age). The IgM immunofluorescence titers were determined to be 1:2 and 1:16 for the children and 1:8 for the adult (age, 60 y). The children with active pinta infection were negative in the IgM FTA-ABS. All reactive sera were negative in tests for rheumatoid factor, a result ruling out the possibility of false-positive IgM reactivity [32].

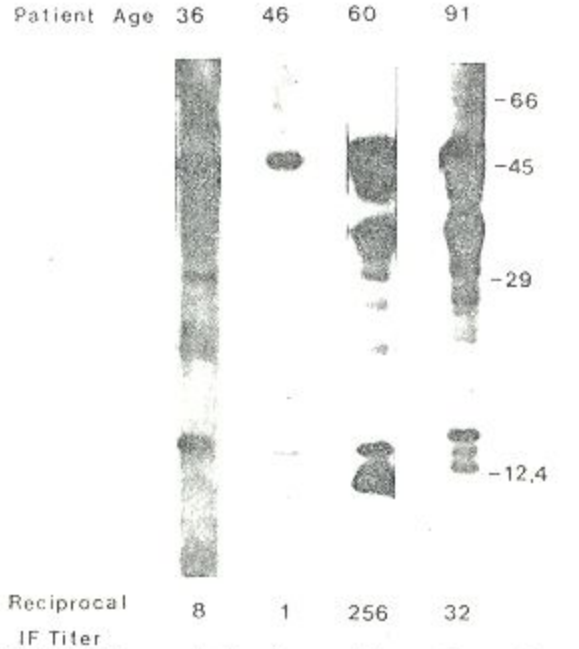

Figure 5. Representative immunoblots of sera from adults (≥19 y of age) with inactive pinta. The patients' IgG immunofluorescence (*IF*) titers and ages are shown. Approximate molecular masses (kDa) are indicated at right.

Discussion

Although the incidence of infection with *T. carateum* has declined as a result of mass treatment campaigns and improved hygiene, pinta still remains a disfiguring disease of clinical and social importance in sev-

eral Central and South American countries, particularly in remote regions. This study characterizes the serological reactivity of children and adults infected with *T. carateum* in commonly used serological tests for syphilis and correlates these results with the presence of antibody to individual treponemal antigens. *T. carateum* fails to survive and multiply in any animal model; therefore, homologous organisms were unavailable for examining antibody development in pinta. Because previous examinations have revealed virtually complete antigenic identity [20, 23, 24] between *T. pallidum* ssp. *pallidum* (Nichols strain) and *T. pallidum* ssp. *pertenue* (yaws; Gauthier strain) and because serological cross-reactivity has been demonstrated between all of the pathogenic treponemes, antibody reactivity in patients with pinta was evaluated against individual polypeptides of *T. pallidum*.

Patients with active and inactive pinta are reactive in both treponemal and nontreponemal tests for syphilis [5–8]. Reactivity in these tests indicates a current or past treponemal infection and assists in the diagnosis of suspected pinta [5–7] when syphilis can be ruled out. In this study, sera from individuals of all ages with clinical evidence of pinta were reactive in both nontreponemal and treponemal tests; no evidence of syphilis was present in this population. In addition, 33 subjects without clinical signs of infection were serologically reactive for syphilis, a result indicating inapparent infection.

Immunoblots of *T. pallidum* ssp. *pallidum* antigens that were reacted with sera from four patients with clinically active pinta revealed a strong similarity to the range of serum reactivity observed in early-active syphilis infection. Reactivity to the 47–48-kDa protein is the most prominent characteristic; other bands, including the 12- and 14-kDa antigens, are seen to a lesser degree [15, 18]. Serum from the oldest (age, eight years) actively infected patient identified at least 10 bands; sera from the youngest (age, one year) identified only one band (47–48 kDa) weakly. The trend of increasing reactivity to treponemal molecules with age in patients with active disease may indicate the length of infection. In primary syphilis, such a correlation exists [18]; increasing reactivity is a direct reflection of the duration of symptoms. Unlike syphilis infection, however, active pinta lesions may persist or recur for many years.

Sera from children with inactive pinta contained antibodies to the full spectrum of *T. pallidum* antigens, whereas only seven of the 11 adult sera were reactive. The range of antibody reactivity seen in sera from young patients with inactive disease closely resembled that seen in patients with early-latent syphilis [15–18]; antibody reactivity was strong and was directed against many treponemal antigens. In late-latent syphilis, the intensity of antibody staining and the spectrum of reactivity decreases with longer duration of the latent state [15–18]. Similarly, many adults with presumably long-standing, latent pinta infection demonstrate loss of antibody reactivity to several treponemal antigens and overall decreased intensity of staining in immunoblots. Continued high levels of antibody reactivity in immunoblots, as well as VDRL and FTA-ABS titers, among certain members in the adult population of the village may indicate chronic reexposure to *T. carateum* by contact with infected children.

Although the pathogenic treponemes are morphologically identical and produce chronic granulomatous diseases characterized by active and latent stages, the epidemiology and clinical manifestations of the infections are very different. Molecular comparisons of *T. pallidum* ssp. *pallidum* and *T. pallidum* ssp. *pertenue* have revealed only subtle differences between the two organisms [20, 23, 24], and DNA homology studies show virtual genetic identity [1]. The degree of antigenic relatedness of *T. carateum* to the other treponemes has not been systematically examined at the molecular level. The observation of Radolf et al. [33] that sera from most patients infected with pinta and yaws are reactive to the recombinant 4D antigen of *T. pallidum* ssp. *pallidum* (Nichols strain) suggests that *T. carateum* and *T. pallidum* ssp. *pertenue* contain at least one antigenically similar molecule. Our study demonstrated that patients with pinta developed antibody reactivity to the full spectrum of antigenic molecules of *T. pallidum* ssp. *pallidum*, an occurrence indicating a very high degree of antigenic relatedness for the two organisms. The development of antibody to these individual molecules seems to follow a pattern similar to that seen in patients with syphilis and suggests that the expression of certain epitopes may be similar. These antigens also include several (47–48, 37, 14, and 12 kDa) that have clearly been shown to possess epitopes specific for the pathogenic treponemes [27, 34]. The confirmation of molecular antigenic similarity between *T. carateum* and the other pathogenic treponemes reaffirms the validity of using *T. pallidum* ssp. *pallidum*, Nichols strain, as the strain type for examining the pathogenic treponemes.

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