Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis Immunoblotting as a Serological Tool in the Diagnosis of Syphilitic Infections

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The utility of sodium dodecyl sulfate-polyacrylamide gel electrophoresis immunoblotting as a serological tool in the diagnosis of human syphilitic infections was examined. In model experiments, rabbits were immunized with *Treponema pallidum* or *T. phagedenis*, and the antisera were tested for cross-reactivities with both sets of antigens. A major *T. pallidum* antigen with a molecular weight of ca. 17,000 appeared to be the most reliable specific antigenic marker as assessed by the immunoblotting technique with peroxidase-labeled second antibodies. Antigens to this antigen were never detected in hyperimmune rabbit anti-*T. phagedenis* sera or in the sera of nonsyphilitic humans. In contrast, reactive antibodies were found in all syphilitic human sera and also in liquor samples that were positive in the passive hemagglutination test. Differentiation between immunoglobulin M and immunoglobulin G antibodies was directly possible by applying the respective specific second antibodies. Immunoblotting tests were performed with sera exhibiting low passive hemagglutination test titers and equivocal fluorescent treponemal antibody and rapid plasma reagin card reactions. In more than 60% of these cases, immunoblot positivity with respect to the 17,000-molecular-weight antigen was found. The same results were obtained with partially purified 17,000-molecular-weight antigen. The immunoblot technique should be useful as an additional diagnostic tool for differentiating between true and false-positive serological reactions.

The analysis of the humoral response to protein antigens of *Treponema pallidum* and *T. phagedenis* has made great progress through the recent application of immunoblotting and electroimmunoassay techniques (1, 2, 4–6, 8–12). Western blotting with 125I-protein A led to the definition of 8 (8) to 22 (6) *T. pallidum* antigens reacting both with rabbit and human antibodies (4, 5, 8). Cross-reactions between antibodies to *T. phagedenis* and several *T. pallidum* antigens have been previously reported (4, 8, 12). Although *T. pallidum*-specific antigens do not seem to exist, general consensus has not yet been reached regarding their number and molecular size. Hanff et al. (5) defined 14 *T. pallidum*-specific polypeptides, whereas Lukehart et al. (8) described 3 specific bands with molecular weight (MW) of 12,000 (12K), 14K, and 48K. Three specific antigens were also identified by Pedersen et al. by electroimmunoassays (10, 12). In all cases, normal human sera were reported not to contain antibodies to these moieties.

The objective of the present study was to examine the potential usefulness of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) immunoblot system for aiding the diagnosis of human syphilitic infections. We used peroxidase staining of immunoblots and first examined the cross-reactivities of rabbit antisera to *T. pallidum* and *T. phagedenis* obtained by experimental infection or immunization. We found that a paired band of *T. pallidum* antigens with MW of ca. 14K and 17K probably represent an easily purifiable, specific determinant for immunoblot analyses. Antibodies to these antigens became detectable after day 10 of experimental syphilitic infections and persisted over the entire period of observation (1.5 to 2 years). The 14K and 17K antigens were not recognized by hyperimmune rabbit antisera to *T. phagedenis* or by normal human sera. In contrast, the antigens were recognized without exception by human antibodies in 130 syphilitic sera examined. The immunoblot procedure is rapid and simple enough to be performed in routine serological laboratories and readily permits differentiation between immunoglobulin M (IgM) and IgG antibodies in unfractionated sera. Its use is advocated particularly in cases in which the "classical" passive hemagglutination test (TPHA) and fluorescent treponemal antibody-absorbed (FTA-abs) reactions yielded borderline, equivocal results.

**MATERIALS AND METHODS**

**Antigen preparations.** Adult male rabbits were used for the passage of *T. pallidum*. All preimmune sera were found to be free of antibodies against treponemes. Rabbits were infected intratesticularly with *T. pallidum* (Nichols) and treated with prednison (1 mg/kg) on day 3, 5, 7, and 9 postinfection. When orchitis developed, the rabbits were sacrificed with a lethal injection of pentobarbital sodium. Testes were removed aseptically, sliced, and washed repeatedly with 10 ml of RPMI 1640 medium (Flow Laboratories, Irvine KA 12 8 NB, Scotland). Cellular debris was removed by centrifugation at 1,400 × g for 30 min at 4°C. The supernatants were decanted, pooled, and centrifuged at 16,000 × g for 20 min at 4°C. The pellets containing the treponemes were washed thrice with 0.2 M phosphate-buffered saline (pH 7.4) containing 1 mM dithioerythritil. The treponemes were finally concentrated ca. 100-fold to the primary volume by centrifugation.

Suspensions of *T. phagedenis*, biotype Reiter, were prepared by cultivation in Spirobroth (BBL Laboratories, Cockeysville, Md.) supplemented with 10% heat-inactivated normal rabbit serum at 37°C for 5 days under anaerobic conditions in the dark. Organisms were harvested by centrifugation at 16,000 × g for 20 min and washed thrice with phosphate-buffered saline–1 mM dithioerythritil. To all prep-
Rabbit immune sera. Syphilitic rabbit sera were obtained from rabbits infected as described above at day 10 and later postinfection. These rabbits received no prednisin. Some rabbits were boostered with washed treponemes (1 mg/ml) suspended in saline and admixed with 1 volume of Freund incomplete adjuvant (Difco Laboratories, Detroit, Mich.). Antisera against T. phagedenis were obtained by repeated immunization with washed T. phagedenis (1 mg/ml) admixed with 1 volume of incomplete Freund adjuvant. Antigen was administered intracutaneously every second day during week 1 and a total of three booster injections were generally given at time intervals of 2 to 3 weeks. Hyperimmune sera were obtained on day 5 after the final boost.

Human serum and liquor samples. Serum and liquor samples were obtained from the serological laboratory of our institute. Rapid plasma reagin (RPR), FTA-abs and TPHA titers were determined by routine procedures. The results of the FTA-abs assays were expressed as (+) to indicate borderline reactions or as +, ++, and +++ to indicate one, two, and three to four positive reactions, respectively. Serological positive sera were obtained from blood donors with no reactions, and three to four positive reactions, respectively.

Gels were calibrated with the Pharmacia (Uppsala, Sweden) low-molecular-weight marker kit containing (94K), bovine serum albumin (67K), carbonic anhydrase (30K), soybean trypsin inhibitor (20.1K), and α-lactalbumin (14.4K). Gels were stained for 2 h at room temperature in 0.2% Coomassie blue R 250 in 50% ethanol–10% acetic acid and destained for 1 to 2 h in 50% ethanol–10% acetic acid, followed by several hours in 10% acetic acid.

Electrophoretic transfer of proteins from the gels to nitrocellulose paper (13) (0.45 μm; Sartorius, Göttingen, Federal Republic of Germany) was performed for a minimum of 3 h at 0°C at a voltage of 130 V. The buffer was 8 mM Tris–64 mM glycine–20% (vol/vol) methanol. Transfer was performed by staining the paper blots with amido black, and the blotted gels were also stained subsequently with Coomassie blue. After protein transfer, the blots were incubated for 30 min in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl [pH 8.5]) containing 0.5% (vol/vol) Tween 20 to block unoccupied sites. The blots were then transferred to TBS buffer containing 0.05% Tween 20, and the serum to be tested was added in a final dilution of 1:10 or 1:100. The total volume of each serum assay was 6 to 8 ml. All incubations were performed at room temperature under gentle agitation. After overnight incubation with the antisera, the blots were washed four times in TBS–0.05% Tween 20 and then incubated with peroxidase-conjugated second antibodies (90 min; dilution, 1:500). The following antibodies were used: swine anti-rabbit IgG; polyvalent rabbit anti-human IgG, IgM, and IgA; rabbit anti-human IgG (γ-chain specific); and rabbit anti-human IgM (μ-chain specific) (all from Dakopatts Immunglobulins, Copenhagen, Denmark). The immunoblots were then washed thrice with TBS–0.05% Tween and transferred to 10 mM Tris-hydrochloride (pH 8.0) containing no detergent. They were developed with a solution of 20 mg of 3-amino 9-ethylcarbazole (dissolved in 2 ml of dimethylformamide) in 50 ml of 0.1 M Tris-hydrochloride (pH 8.0) given 30 μl of 30% H2O2. After development, the blots were rinsed with TBS buffer and photographed.

Partial purification of the 17K antigen from T. pallidum. T. pallidum suspensions in 0.2 M phosphate-buffered saline–1 mM dithioerythrit–1 mM polymethylsulfonyl fluoride were incubated for 1 h with 1% (vol/vol) Triton X-100. The suspensions were centrifuged at 30,000 × g for 15 min, and the supernatants were recovered. The antigen solutions were then equilibrated in 50 mM NaCl–25 mM Veronal (pH 7.0) by a passage through Sephadex G-25 (PD 10 columns; Pharmacia) and applied to a DEAE-Sephacel column (5 by 1.2 cm) equilibrated in this buffer. The 17K antigen passed the column and was collected in a total of 8 ml after a column wash with 10 ml of buffer. The antigen preparations were concentrated 10-fold with immersible CX-10 single-use ultrafiltration units (Millipore Corp., Bedford, Mass.) and then chromatographed over an ACA 44 column (90 by 2.5 cm; LKB Laboratories, Bromma, Sweden) in 50 mM Tris–0.2 M NaCl (pH 9.0). Fractions of 1 ml each were collected, and those containing the 17K antigen as detected by immunoblotting were pooled and used as the test antigen.

RESULTS

SDS-PAGE immunoblotting of T. pallidum antigens developed with hyperimmune rabbit antisera to T. pallidum and T. phagedenis. Figure 1 depicts SDS-PAGE patterns of T. pallidum antigens obtained by Coomassie staining (lane a) and by immunoblotting with hyperimmune rabbit antisera to T. pallidum (lane b) and T. phagedenis (lane c). The results were similar to those reported by Hanff et al. (4) and Lukehart et al. (8). However, the number of T. pallidum antigens cross-reacting with anti-T. phagedenis antibodies is shown on the left. (b) SDS-PAGE immunoblot pattern of T. pallidum antigens developed with the homologous rabbit hyperimmune anti-T. pallidum serum. (c) SDS-PAGE immunoblot pattern of T. pallidum antigens developed with rabbit hyperimmune anti-T. phagedenis serum. The latter serum recognized a multitude of T. pallidum antigens but did not stain the 14K to 17K moieties.

FIG. 1. (a) SDS-PAGE pattern of T. pallidum polypeptides after electrophoresis on a 12.5% gel and staining with Coomassie brilliant blue. The positions of the molecular weight markers are shown on the left. (b) SDS-PAGE immunoblot pattern of T. pallidum antigens developed with the homologous rabbit hyperimmune anti-T. pallidum serum. (c) SDS-PAGE immunoblot pattern of T. pallidum antigens developed with rabbit hyperimmune anti-T. phagedenis serum. The latter serum recognized a multitude of T. pallidum antigens but did not stain the 14K to 17K moieties.
The 14K and 17K antigens appeared to be easily definable, specific immunogens of *T. pallidum*, since they did not stain with high-titered antisera to *T. phagedenis* (Fig. 1). To determine the stage at which antibodies to these moieties would become detectable, we tested sera of rabbits after various times postinfection. Whereas preimmune serum never showed 17K reactivity, antibodies to these antigens were detectable already at day 10 postinfection (Fig. 2). Titters increased thereafter and persisted throughout the entire course of our experiments (1 to 2 years). Preimmune rabbit sera sometimes recognized 90K, 60K, 42K, and 30K antigens of *T. pallidum*, and such nonspecific staining patterns resembled those frequently observed with nonsyphilitic human sera (see below).

**T. pallidum** antigens recognized by syphilitic human sera. A total of 130 sera exhibiting TPHA titers of $\geq 1:256$ and positive FTA_{abs} and RPR reactions were examined. The sera comprised seven groups, i.e., (i) 3 cases of treated, primary syphilis, (ii) 6 cases of untreated secondary syphilis, (iii) 29 cases of treated secondary syphilis, (iv) 10 cases of strong seropositivity without typical clinical symptoms of syphilis, (v) 1 case of tabes dorsalis, (vi) 1 case of neurosyphilis, and (vii) 3 cases for which a precise clinical history could not be obtained.

Irrespective of the clinical stage of infection, the 17K antigen was recognized by these sera without exception; the 14K antigen showed some variation. Sera developed with anti-IgG second antibody that reacted strongly with the 14K and 17K antigens generally showed strong accompanying reactions, with two additional doublets of ca. 40K and 43K and 54K and 60K and, more variably, with bands between 43K and 54K MW. These results are basically consistent with the report of Hanff et al. (5), although the differences reported to exist among human sera at different stages of syphilitic infection were not so pronounced as to allow a distinct classification in our experience. The major emphasis of this study is placed on the recognition that the 17K moiety probably represents the most reliable single antigenic marker in human syphilitic infections. The slight variations in apparent MW of other antigens compared with those given in previous studies (4–6, 8) are likely to derive from methodology and are not regarded as basically relevant.

The immunobLOTS of Fig. 3 were prepared with *T. pallidum* as well as *T. phagedenis* antigens (lanes a and b, respectively). Many *T. phagedenis* antigens were recognized especially by the high-titered syphilitic sera. Of these, one *T. phagedenis* antigen of ca. 14K and 15K MW also stained occasionally. This finding should not be taken as a contradiction to the contention that the 14K and 17K *T. pallidum* antigens are species specific, since similar migration in SDS-PAGE cannot be equated with immunological identity. The 14K and 15K *T. phagedenis* antigen is, indeed, probably not antigenically related to its counterparts in the *T. pallidum* system, since high-titered rabbit antisera prepared to *T. phagedenis* stain the homologous antigen (data not shown) but do not stain the respective *T. pallidum* antigens (Fig. 1).

Differentiation between IgG and IgM antibodies in unfractionated sera were simply and directly achieved by using the appropriate commercially available second antibodies. Sera positive for IgM in the FTA_{abs} test were also IgM positive for the 17K antigen in the immunoblot; no exceptions were ever noted. Figure 4 depicts the identification of IgM antibodies in four different sera of patients with untreated secondary syphilis. Additional experiments were also performed in which the IgM fractions were recovered after separation for IgG by sucrose density gradient centrifugation, and the same results were obtained (data not shown).

**Analysis of liquor samples.** We analyzed a total of 30 different liquor samples. Nine were from nonsyphilitic patients and served as controls. Six samples were from patients exhibiting seropositivity in the FTA_{abs}, TPHA, and immunoblot tests, but whose liquor samples were negative in the former assays. No positivity in the staining of the 14K and 17K moieties was found in any of these 15 cases with the immunoblot technique (data not shown). In contrast, 12 liquor samples with TPHA titers ranging from 1:64 to 1:512 all recognized the 14K and 17K antigens, and the respective antibodies were identified as IgG (Fig. 5). IgM antibodies were not detected in any of these samples. Three final liquor samples that were judged positive by the immunoblot assay were of particular interest, because these samples had been

![FIG. 2. Antibody response of two infected rabbits to *T. pallidum* polypeptides at day 10 (a and b) and month 3 (c and d) postinfection. The antisera had already recognized the 17K *T. pallidum* antigen at day 10 after infection.](http://jcm.asm.org/)

![FIG. 3. Typical immunoblot patterns of human syphilitic sera (A to D) that showed different reactions in the TPHA, FTA_{abs}, and RPR tests. Serum was from a patient with primary syphilis (A) and sera from patients with secondary syphilis (B to D) are shown. *T. pallidum* extracts (lanes a) and *T. phagedenis* extracts (lanes b) were applied. All syphilitic sera recognized the 17K *T. pallidum* antigen. The sera also reacted with some antigens of *T. phagedenis*.](http://jcm.asm.org/)
graded negative in the classical TPHA, FTA_{abs}, and RPR tests. One sample was from a patient with tabes dorsalis, one was from a patient with lues latens seropositive, and one was from a lues seropositive acquired immunodeficiency syndrome patient.

Reactions of serologically negative human sera. Hanff et al. (6) have reported the reaction of nonspecific human antibodies present in the sera of healthy individuals with four common antigens of T. pallidum and T. phagedenis (MW of 30K, 33K, 40K, and 45K), of which two may represent axial filament proteins. We examined a total of 30 different sera and a serum pool from 100 donors that were negative in TPHA and FTA_{abs} tests. In some cases, we found no reactivities with T. pallidum antigens; the immunoblots were entirely blank. In other cases, reactions with 43K and 54K antigens occurred; slight staining was also sometimes detected on 30K and 90K bands (Fig. 6). In contrast to Hanff (5) and Lukehart (8), we often found a remarkably strong reaction with the 60K antigen (Fig. 6, lanes e, f, and g). Thus, the 60K antigen, although strongly reactive with all syphilitic sera tested, does not appear to represent a specific antigenic marker for T. pallidum. The 17K antigen was, in contrast, never detected by any of the negative sera.

Immunoblotting results obtained with sera exhibiting borderline serological reactions. The above results led us to suspect that the 17K antigen probably represents the most reliable single antigenic marker for diagnosis of human syphilitic infection. Clear positivity with respect to this moiety was tentatively taken to indicate the presence of truly specific antibodies to T. pallidum. Applying this criterion, we then examined 147 sera that exhibited borderline serological reactions. Cases included sera that showed isolated TPHA titers of 1:64 and 1:128 (negative FTA_{abs} and RPR titers), and cases exhibiting equivocal RPR, FTA_{abs}, or both reactions. The sera were grouped into five categories (Table 1). More than 60% of the sera developed with polyvalent anti-IgA, IgM, and IgG antibodies clearly recognized the 17K antigen; this generally coincided with faint recognition of other bands, in particular the 54K and 60K doublet, that constituted the typical pattern of positive syphilitic sera (Fig. 7; cf. Fig. 3). These almost certainly represented true syphilitic sera. The other sera did not recognize the 17K antigen and showed varying recognition of other bands (Fig. 7, lane a). These sera were provisionally classified as false-positive. In one case, a series of serum

![FIG. 4. Detection of IgM antibodies to T. pallidum antigens in unfractionated human sera. T. pallidum extracts were electrophoresed, and immunoblots were developed with the sera of four patients (A to D) exhibiting positive IgM titers. The second antibodies used were rabbit anti-human immunoglobulins (IgG, IgM, and IgA; lanes a) and specific rabbit anti-human IgM (lanes b). Note the detection of IgM antibodies present against certain T. pallidum antigens, including the 17K moiety.](http://jcm.asm.org/)

![FIG. 5. T. pallidum antigens recognized by antibodies present in five liquor samples of syphilitic patients (a to e). Second antibodies were rabbit anti-human immunoglobulins. The 17K antigen was recognized in all cases, and isolated staining of this moiety was found in one case (d).](http://jcm.asm.org/)

![FIG. 6. T. pallidum antigens detected by nonspecific antibodies present in 10 different negative human sera (a to k). The reaction of a positive serum is shown for comparison (i). None of the negative sera recognized the 17K antigen. The 60K antigen was stained heavily in three cases (e, f, and g).](http://jcm.asm.org/)

**TABLE 1. Immunoblot reactivity of 147 serum samples exhibiting borderline serological reactions with the 17K antigen of T. pallidum**

<table>
<thead>
<tr>
<th>Group</th>
<th>Results with:</th>
<th>No. of samples</th>
<th>No. (%) showing reactivity with 17K antigen</th>
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<tr>
<td></td>
<td>RPR</td>
<td>TPHA (titer)</td>
<td>FTA_{abs}</td>
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<tr>
<td>A</td>
<td>+</td>
<td>1:64/1:128</td>
<td>+</td>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>1:64/1:128</td>
<td>(+)</td>
</tr>
<tr>
<td>C</td>
<td>0</td>
<td>1:64/1:128</td>
<td>+</td>
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<tr>
<td>D</td>
<td>0</td>
<td>1:64/1:128</td>
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<td>E</td>
<td>0</td>
<td>1:64/1:128</td>
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samples was obtained over a period of 3 years from a young female with no history of syphilis, but with a constant TPHA titer of 1:64. The serum of this patient also never recognized the 17K antigen in the immunoblots. A closer inspection of the results with immunoblotting (Table 1) indicates that, as might be expected, 17K positivity was found in virtually all cases in which all three serological reactions (TPHA, FTA-abs, and RPR) were weakly positive. Because one or two of these classical reactions became negative or equivocal, the percentage of cases exhibiting 17K negativity rose. It appears significant that in group E which comprises cases with isolated, low TPHA titers, immunoblot positivity was still clearly detected in 35% of the tested sera.

A clinical correlation could not be obtained for the majority of these patients, because the sera had been selected out of a random collection spanning 4 years solely on the basis of their low seropositivity which were generally accidental findings. However, there were a few interesting exceptions. In groups A and C, two patients were retrospectively found to represent cases of sufficiently treated primary syphilis; their sera contained no IgM antibodies. In groups B and C, there were two cases of old, treated infections (without IgM antibodies) and two cases of fresh, untreated primary syphilis that had been diagnosed clinically. These latter two sera were indeed found to contain specific IgM antibodies by the immunoblotting technique.

**Immunoblotting with isolated 14K and 17K antigen.** A single passage of a Triton X-100 treponemal extract over DEAE-Sephacel at pH 7 led to the recovery of the 14K and 17K antigens in satisfactorily pure form as assessed by immunoblotting. We used this antigen preparation to examine negative and positive sera and consistently found correct reactions (Fig. 8). The amount of purified antigen to be applied was approximated from comparative stainings with defined positive sera, and overloads were avoided to circumvent any danger of nonspecific staining reactions with negative sera. Under these conditions, none of the tested 20 seronegative sera yielded a positive immunoblot, whereas positivities were observed with all tested positive samples (n = 20).

**DISCUSSION**

Our investigations were launched with the primary intent of examining the possible utility of SDS-PAGE immunoblotting in the serological diagnosis of human syphilitic infections. As this work was underway, a number of reports appeared that were, on the whole, consistent with the data obtained here. We utilized peroxidase-labeled second antibodies rather than 125I-protein A since the antibodies allow more complete detection of human IgG antibodies and also permit straightforward differentiation of the antibody classes. The possibility of occasional artifacts generated by rheumatoid factor in the detection of IgM antibodies has not been excluded and may require further investigation. At present, the immunoblot method appears reasonably simple to perform, avoids the use of radioactivity, and is more rapid and probably at least as sensitive as the protein A method. There are distinct advantages over the use of internally labeled *Treponema* antigens (9). The latter method requires the preparation of radiolabeled antigen, which is cumbersome. Moreover, preferential incorporation of a given label into certain antigens would obviously generate serious interpretational difficulties, and sensitivity would be markedly reduced with regard to those antigens which incorporate little label. Such considerations most probably explain the discrepancies between this and the cited study (9). In particular, the dominance of antibodies to the 14K and 17K antigens was not noted with the radioprecipitation assay. We note, however, that our results are in very good accord with those of Hanff et al. (5), who also consistently found a strong antibody response to a 15.5K antigen of *T. pallidum* in human sera during all stages of syphilitic infections with the immunoblot method.

The collective data obtained from a study with a large number of syphilitic sera now lead us to conclude that antibodies to the low-MW 14K and 17K *T. pallidum* antigens indeed represent most reliable immunological markers for human syphilitic infections, irrespective of clinical stage. Thus, the hyperimmune rabbit antisera to *T. phagedenis*, although cross-reacting with a multitude of at least 14 antigens of *T. pallidum*, did not react with the 17K antigen. Second, rabbits experimentally infected with *T. pallidum* showed positive immunoblot reactions with sera from rabbits infected with *T. phagedenis*.
positive reactivity (defined as the appearance of a clearly visible band on the nitrocellulose blots) already commencing at day 10 postinfection and persisting throughout the course of our studies. Moreover, sera obtained from seven patients with treated or untreated primary syphilis also exhibited positive reactions. Third, all human syphilitic sera tested, as well as 15 syphilitic liquor samples, reacted with this antigen. Positivity to the 17K moiety was accompanied by a typical pattern comprising four bands of ca. 40K, 43K, 54K, and 60K MW. Data of Hanff et al. (5) have previously suggested an existing correlation between the immune response to these antigens and the stage of syphilitic infection. Our present analyses of the IgG responses have not yielded such clear-cut patterns, and this issue has not been pursued further in our laboratory. Finally, 17K reactivity was not found in any of the 30 serologically negative sera examined.

The usefulness of immunoblotting in the routine serological laboratory became apparent when sera exhibiting borderline reactivities in the classical reactions were examined. These comprised five categories (Table 1). Immunoblot positivity with respect to the 17K antigen was found in virtually all cases in which three classical reactions were slightly positive. If RPR or RPR and FTA-abs reactions were negative, the percentage of serum samples not recognizing the 17K antigen increased. Negativity with regard to this antigen was generally accompanied by nonreactivity towards the 40K, 43K, 56K, and 60K pattern typically recognized by antibodies of syphilitic patients. It is for the evaluation of such sera showing equivocal TPHA, RPR, and FTA-abs reactions that we would advocate the use of the immunoblotting method. Whole extracts of T. pallidum can be used for these analyses. However, it may turn out to be more convenient to work with isolated antigen preparations, e.g., in an immunodot enzyme-linked immunosorbent assay. Initial results with partially purified 14K and 17K antigen preparations have been encouraging. Nevertheless, use of the whole SDS extracts may be required in cases of weak 17K positivity. In such cases, positivity with respect to the 54K and 60K and the 40K and 43K doublet should be taken as an additional criterion in the evaluation. Interpretation of the results should be unidirectional until otherwise proven by an extended series of analyses. Thus, we provisionally grade 17K positivity as reflecting the presence of specific antibodies to T. pallidum, but do not yet conclude that negativity proves their absence. Based on this criterion, a positive reaction in 64 of the 147 borderline cases was found. Of these, two patients had just been clinically diagnosed as having primary syphilis, and four were retrospectively found to have been treated for syphilis in the past. It is clear that an assay for IgM antibodies is essential for diagnosing fresh infections and, indeed, these antibodies were found in the two cases of fresh infections cited above. In conclusion, we believe that the application of the immunoblot technique provides a significant extension over existing serological methods for the diagnosis of human syphilitic infections.

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