Enzyme-Linked Immunosorbent Assay for Detection of Immunoglobulin M Antibody Against the Reiter Treponeme Flagellum in Syphilis

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An enzyme-linked immunosorbent assay (ELISA) for detection of immunoglobulin M (IgM) antibodies against the periplasmatic flagellum of the Reiter treponeme is described. IgM in the test samples was bound to anti-IgM-coated microtest plates, and flagellum-specific IgM antibody was subsequently detected by incubation with a purified flagellum preparation and monospecific anti-flagellum conjugate. Rheumatoid factor, antinuclear antibodies, or flagellum-specific IgG did not interfere. The specificity of the ELISA for IgM antibodies was 99.5% for sera from 200 blood donors and 98.6% for 147 patient sera that gave false-positive reactions in other syphilis serological tests. The sensitivity was 88.5% for sera from 87 patients with first-time primary syphilis, 93.5% for sera from 62 patients with first-time secondary syphilis, 21.4% for sera from 42 patients who were reinfect ed, and 0% for sera from 13 patients with late syphilis. Of the sera from 153 patients with treated syphilis, 7.2% had IgM antibodies, and sera from patients with primary or secondary syphilis generally had no IgM antibodies 6 months after treatment. The finding of IgM antibodies indicates that patients should receive antisyphilis treatment if they have not been treated recently, but a negative result does not exclude the possibility of active syphilis. The method may prove useful for the diagnosis of congenital syphilis in newborns.

Detection of immunoglobulin M (IgM) antibodies against treponemal antigens is important for the diagnosis of congenital syphilis (1, 7, 21) since IgM antibodies do not cross the intact placenta and the presence of IgM antibodies against treponemal antigens in newborns indicates active syphilis. The early appearance of IgM antibodies during infection suggests that the detection of IgM antibodies could also be useful as an early test for syphilis, especially in the serological surveillance of contacts of syphilis patients. IgM antibodies persist for shorter periods after treatment than do IgG antibodies. In theory, IgM antibodies are therefore useful for indicating whether active disease is present or has recently been present in a person with IgG antibodies.

Currently, the most common method for the detection of anti-treponemal IgM antibodies is the IgM fluorescent treponemal antibody absorption (FTA-ABS) test (1). However, as in other indirect fluorescent-antibody tests for IgM antibodies, false-negative and -positive results may occur (4, 10, 16). For increasing the specificity and sensitivity of the IgM FTA-ABS test, separation of IgG and IgM in sera by size chromatography or gradient centrifugation (10, 11) and subsequent testing of the fractions by IgM FTA-ABS test have been recommended.

Elsewhere, we described an enzyme-linked immunosorbent assay (ELISA) for IgG antibodies against the periplasmatic flagellum (axial filament) of the Reiter treponeme and showed that this method has a sensitivity and specificity comparable to those of the FTA-ABS test (15a). Compared with the FTA-ABS test, the IgG ELISA has several advantages: it uses a purified, well-defined antigen from an in vitro cultivable treponeme, it does not involve preabsorption of sera, it is quantitative, it gives results that are read objectively, and it is less laborious.

In the present study, we developed, on basis of the principle originally described by Duremeyer and van der Veen (5), an ELISA for IgM antibodies against the flagellum. This method has the same technical advantages over the FTA-ABS test as does the IgG ELISA, and in
addition, laborious serum fractionation is unnecessary.

**MATERIALS AND METHODS**

**Flagellum antigen.** Flagellum from *Treponema phagedenis* biotype Reiter were purified as previously described (18). The protein concentration of the antigen preparation was 0.3 g/liter, as determined by the method of Lowry et al. (8).

**Rabbit antiflagellum antibody conjugate.** Monospecific antisera against the flagellum preparation were obtained by immunization of three rabbits with immune precipitates of the antigen cut out of unstained, crossed, immunoelctrophoretic plates. Reiter treponeme supernatant obtained after sonication was used as the antigen, and a selected serum from a *T. pallidum*-infected rabbit was used as the antisera (18). IgG was purified from a pool of sera monospecific for the flagellum preparation, and 52 mg of IgG was conjugated to horseradish peroxidase by the two-step glutaraldehyde procedure (3). The conjugate (43 ml) was stored at -20°C until use.

**Solid-phase anti-IgM ELISA for IgM antibodies against the flagellum.** Flat-bottom polystyrene microtest plates (Immunoplates [code no. 2-39454]; Nunc, Roskilde, Denmark) were used as the solid phase for absorption of rabbit anti-human IgM (code no. 10-091; DAKO-Immunoglobulins, Copenhagen, Denmark) diluted 1:1,000 in phosphate-buffered saline (PBS), pH 7.4. A 50-μl portion of anti-human IgM was added to each well, and the plates were incubated overnight at 4°C. The contents of the wells were aspirated, and the wells were washed three times with PBS-0.05% (wt/vol) Tween 20 and then coated with 100 μl of PBS-15% (wt/vol) bovine serum albumin for at least 2 h at room temperature or until the plates were used, generally within 1 week. The wells were washed as described above, and 50 μl of the serum to be tested, diluted 1:200 in PBS-Tween 20-0.5% bovine serum albumin, was added to each of four wells. Incubation was for 4 h at room temperature. The wells were washed, 50 μl of flagellum antigen, diluted 1:25 in PBS, was added to two of the four wells, and 50 μl of PBS was added to the other two wells. After overnight incubation at 4°C, the wells were washed, and 50 μl of conjugate, diluted 1:300 in PBS-Tween 20-20% normal-rabbit serum, was added to each well. After 3 h, the wells were washed, and 200 μl of substrate (41 mg of orthophenyl diamine [Sigma Chemical Co., St. Louis, Mo.], 100 ml of citrate buffer [pH 5], 25 μl of 30% H₂O₂) was added to each well. After incubation for 35 min at room temperature, the reaction was stopped by the addition of 50 μl of 3 M H₂SO₄ to each well. The absorbance was read at 492 nm with a Titertek Multiscan photometer (Flow Laboratories, Inc., Rockville, Md.). Each plate included five standard sera covering the range of absorbances obtained in the assay.

**ELISA for IgG antibodies against the flagellum.** The indirect ELISA for IgG antibodies was performed as described elsewhere (15a).

**Serological tests for rheumatoid factor and antinuclear antibodies.** Rheumatoid factor was detected by the Rose-Waaler Test (17, 25) and the latex agglutination test (22). An immunofluorescence method was used for the detection of antinuclear antibodies (6). Sera with titers of ≥1:40 were regarded as reactive in the tests for rheumatoid factor. A titer of ≥1:10 was regarded as reactive in the test for antinuclear antibodies.

**Routine syphilis serological methods.** Sera from blood donors were tested by the automated reagent test (23), and all other sera were tested by the cardiolipin Wassermann test as modified by Mörch (CWRM) (20) and by the Kahn standard test (KR) (20). The results of CWRM and KR (STS) were recorded as positive when a serum was reactive in one or both tests. All sera were analyzed by the FTA-ABS test (25), and practically all were analyzed by the *T. pallidum* immobilization (TPI) test (14) as well. The conjugate used in the FTA-ABS test was a rabbit anti-human IgG, -A, -M antisemur (code F1009; DAKO-Immunoglobulins).

**Diagnoses of syphilis.** The diagnoses of syphilis were made on basis of extensive, centralized, serological and clinical data collected at the Department of Treponematoses, Statens Seruminstitut, Copenhagen.

**Sera.** Patient sera were chosen from specimens submitted for routine syphilis serological analysis during a 6-month period in 1980. From each patient except those in group 10 (see below), one serum sample was obtained. All sera were stored at -20°C until use. Most of the sera were from another study (15a) and were obtained from one of the following groups.

**Group 1: patients with untreated primary syphilis.** There were 87 patients with primary untreated syphilis. All patients had lesions suspected of being chancre, and all but four had sera that were reactive in STS or had treponemal antibodies found by dark-field microscopy. Of the four patients with nonreactive sera in STS and for whom dark-field microscopy was not done or was done with a negative result, all gave succeeding samples that were reactive in STS. All group 1 patients were considered to have syphilis for the first time.

**Group 2: patients with untreated secondary syphilis.** There were 62 patients with untreated secondary syphilis. All patients had exanthema, and all gave sera that were reactive in STS. We believed that these patients had syphilis for the first time.

**Group 3: patients with untreated syphilitic reinfections.** There were 42 patients with syphilitic reinfections. All patients had untreated primary syphilis (17 cases) or untreated secondary syphilis (25 cases). All patients had records of previously diagnosed and treated syphilis.

**Group 4: patients with untreated late syphilis.** There were 13 patients with untreated late syphilis. Four patients had late latent syphilis, and nine patients had tertiary syphilis. All sera were reactive in STS and the TPI and FTA-ABS tests. None of the patients had been previously treated for syphilis.

**Group 5: patients with treated syphilis.** There were 153 patients with treated syphilis. The stages at the time of diagnosis differed, and both early and late syphilis cases were represented. The median time that had elapsed since diagnosis was 3.0 years (range, 2 months to 69 years).

**Group 6: persons whose sera gave false-positive reactions.** There were 147 persons whose sera gave false-positive reactions, including persons whose sera were considered to have or have had false-positive reactions in STS (133 cases), the TPI test (6 cases) or the FTA-ABS test (8 cases). For each serum, reactivity was
found in no more than one treponemal test. None of the persons had any history or signs of syphilis. Forty-five group 6 persons had sera that chronically gave false-positive reactions (i.e., sera that were serologically reactive for more than 6 months), 18 had sera that gave false-positive reactions acutely, and 84 were not followed long enough to ascertain whether their sera gave false-positive reactions acutely or chronically.

**Group 7: Persons without syphilis.** There were 200 sera from unpaid voluntary blood donors without syphilis. All sera were nonreactive in the automated reagin test, and none of the persons in this group had a history of past or present syphilis.

**Group 8: Persons with rheumatoid factor-positive sera.** We randomly selected 25 sera among specimens found to be positive for rheumatoid factor by the Laboratory for Autoimmune Diseases, Statens Serum Institut. The sera contained rheumatoid factor in titers ranging from 1:40 to ≥1:5,120, as determined by the Rose-Waaler test. Eight sera contained antinuclear antibodies in titers ranging from 1:40 to ≥1:1,280.

**Group 9: Newborns.** We obtained 25 sera from newborns. All sera were reactive in the FTA-ABS test and the IgG ELISA. None of the children had clinical signs of congenital syphilis. The mothers had been treated for syphilis before or during pregnancy.

**Group 10: Patients with primary and secondary syphilis who gave consecutive samples.** A total of 24 sera were collected from seven patients with primary syphilis and three patients with secondary syphilis. All patients had syphilis for the first time.

**Statistical analysis.** The sign test was used for statistical analysis. The level of significance was \( P \leq 0.05 \).

**Specificity and sensitivity of the test.** The specificity was defined as the percentage of unaffected individuals whose sera were nonreactive in the test in relation to the number of unaffected individuals in the population tested. The sensitivity was defined as the percentage of affected individuals whose syphilis was detected by the test in relation to the number of affected individuals in the population tested.

**RESULTS**

**Cutoff point and precision of the anti-IgM ELISA.** To reduce between-assay variation, we adjusted the absorbance value of one of the five reference sera to 0.93 (mean value of 25 assays) in every assay by multiplication with a correction factor. The absorbance values of all sera on the same plate were multiplied by the same factor used for correcting the reference serum value. The total-assay precision of the anti-IgM ELISA is shown in Table 1. The results for 200 sera from blood donors (group 7) are shown in Fig. 1 and Table 2. The absorbance values for 199 of the sera were below 0.35, and this value was chosen as the cutoff value between reactive and nonreactive sera. The single reactive serum from a blood donor (Fig. 1) was not reactive in any of the other syphilis serological tests and did not contain rheumatoid factor or antinuclear antibodies.

**IgM antibodies in sera from patients with syphilis.** The distribution of absorbance values determined by the anti-IgM ELISA is shown in Fig. 1, and the results of all syphilis serological tests are shown in Table 2.

Seventy-seven (88.5%) of the sera from patients with untreated primary syphilis (group 1) were reactive in the anti-IgM ELISA, and six of those were nonreactive in the IgG ELISA. Ten (11.5%) of the sera were nonreactive in the anti-IgM ELISA: of these, four were nonreactive in the IgG ELISA, and six were reactive.

Fifty-eight (93.5%) of the sera from patients with untreated secondary syphilis (group 2) were reactive in the anti-IgM ELISA. The sera of all patients were reactive in the other tests, and the differences in sensitivity between the anti-IgM ELISA and the other tests were not significant.

Only nine (21.4%) of the sera from patients who were reinfected (group 3) were reactive in the anti-IgM ELISA, whereas all were reactive in all of the other syphilis serological tests \((P < 0.01)\).

The sera of all 13 patients with untreated late syphilis (group 4) were nonreactive in the anti-IgM ELISA, but all were reactive in the other syphilis serological methods \((P < 0.01)\).

Eleven (7.2%) patients in the group with treated syphilis (group 5) had sera that were reactive in the anti-IgM ELISA. In comparison, 79.1% of the patients had sera that were reactive in the IgG ELISA \((P < 0.01)\). Eight of the patients with IgM antibodies against the flagellum had been treated for syphilis <10 months previously. One patient was treated 15 months previously, one was treated 30 months previously, and one was treated 33 years previously. The 11 sera reactive in the anti-IgM ELISA were all reactive in the IgG ELISA and the FTA-ABS, and TPI tests. None of the sera had rheumatoid factor. Figure 2 shows the results of the consecutive samples (group 10). Most samples were nonreactive 6 months after treatment.

**Specificity of the anti-IgM ELISA.** Of 147 false-positive sera (from group 6), 2 were reactive in the anti-IgM ELISA. Both of these sera were reactive in the STS but nonreactive in the TPI and FTA-ABS tests, the IgG ELISA, and the tests for rheumatoid factor. The specificity of
the anti-IgM ELISA was significantly higher than that of the IgG ELISA \((P \leq 0.05)\).

We tested 25 sera containing rheumatoid factor (from group 8), and all were nonreactive in the anti-IgM ELISA. The absorbance values obtained without flagellum antigen for sera from group 8 were not higher than the values obtained without flagellum antigen for sera from the blood donor group (group 8). If the rabbit serum was omitted from the conjugate, the absorbance value obtained without the flagellum antigen increased, and one of the rheumatoid factor-positive sera (Rose-Waaler titer, 1:5,120) became reactive in the anti-IgM ELISA. This serum also

### Table 2. Results of syphilis serological tests of sera from controls and from patients with different stages of syphilis

<table>
<thead>
<tr>
<th>Patient group</th>
<th>No. of sera</th>
<th>% of sera showing indicated result</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>STS</td>
</tr>
<tr>
<td>1. Untreated primary syphilis</td>
<td>87 (84)</td>
<td></td>
</tr>
<tr>
<td>2. Untreated secondary syphilis</td>
<td>62 (61)</td>
<td>0.0</td>
</tr>
<tr>
<td>3. Untreated primary or secondary</td>
<td>42 (41)</td>
<td>0.0</td>
</tr>
<tr>
<td>syphilis (reinfactions)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Untreated late latent or tertiary</td>
<td>13 (13)</td>
<td>0.0</td>
</tr>
<tr>
<td>syphilis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Treated syphilis</td>
<td>153 (150)</td>
<td>67.3</td>
</tr>
<tr>
<td>6. False-positive reactions</td>
<td>147 (146)</td>
<td>25.9</td>
</tr>
<tr>
<td>7. No syphilis (blood donors)</td>
<td>200 (200)</td>
<td>—</td>
</tr>
</tbody>
</table>

\(a\) Numbers in parentheses indicate numbers of sera tested by the TPI test.
\(b\) Includes borderline reactions.
\(c\), Sera from blood donors were tested by the automated reagin test.
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FIG. 2. Serial determinations of anti-IgM ELISA absorbance values for sera from seven patients with primary syphilis (●) and three patients with secondary syphilis (○). Sera with absorbance values above the dotted horizontal line were regarded as reactive. Each point is the mean value of two determinations. A 492, Absorbance at 492 nm.

contained antinuclear antibodies (titer, ≥1:320).

Three rheumatoid factor-positive sera (Rose-Waaler titer, ≥1:5,120) were diluted 1:100 and mixed in a 1:1 ratio with four sera diluted 1:100 that were reactive in the IgG ELISA and nonreactive in the anti-IgM ELISA. All 12 mixtures were nonreactive in the anti-IgM ELISA.

None of the sera from newborns (group 9) were reactive in the anti-IgM ELISA, but all were reactive in IgG ELISA and the FTA-ABS test.

DISCUSSION

After it was described in 1978 (5), the solid-phase anti-IgM ELISA became the method of choice for the detection of IgM antibodies. The method eliminates competition between IgG and IgM antibodies for antigenic sites. This competition may cause false-negative IgM results both in the indirect ELISA (24) and in the indirect fluorescent-antibody tests (4, 10) such as the IgM FTA-ABS test. The anti-IgM ELISA to a certain degree also eliminates the interaction of rheumatoid factors, which may cause false-positive results in the indirect methods (16). Rheumatoid factors may, however, also interact in the anti-IgM ELISA method (24). The interaction is caused by the binding of IgM rheumatoid factor to anti-IgM antibodies bound to the microtest plate. Rheumatoid factor may be able to bind the conjugate even in absence of antigen. Naot et al. (13) obtained false-positive results only when both rheumatoid factor and antinuclear antibodies were present in the serum. Interactions of rheumatoid factors and antinuclear antibodies were not seen in the present study, not even when rheumatoid factor-containing sera were mixed with sera containing high levels of IgG antibodies against the flagellum. The addition of normal-rabbit serum to the conjugate decreased the nonspecific binding of the conjugate to the plate. A single serum that contained both rheumatoid factor and antinuclear antibodies would have given a false-positive reaction in the anti-IgM ELISA without the addition of rabbit serum.

Most of the sera from patients with untreated first-time primary syphilis (group 1) and secondary syphilis (group 2) were reactive in the anti-IgM ELISA, but most of the sera from patients with untreated reinfections (group 3) and all of the sera from patients with untreated late syphilis (group 4) were nonreactive. For group 3, the high percentage of sera that were reactive in the IgG ELISA and the low percentage of sera that were reactive in the anti-IgM ELISA are characteristic of an anamnestic immune response in which T-cells are involved. The nonreactivity in the anti-IgM ELISA of sera from patients with late syphilis (group 4) can be explained similarly.

IgM FTA-ABS test results are conflicting and only to a certain degree in accordance with the present study. Müller (10) separated IgM and IgG in 1,582 sera from patients with latent, late latent, and neurosyphilis by size chromatography. Of these sera, 25.4% were reactive in the IgM FTA-ABS test before fractionation, and 54.0% were reactive after fractionation. Müller and Oelerich (12) showed that the IgM titers of fractionated sera from patients with late syphilis were lower than the titers of sera from patients with early syphilis and that most sera were reactive; for sera from reinfected patients, nonreactive IgM FTA-ABS test results were often seen. In a study by Atwood and Miller (2), the serum of only one of eight patients with late syphilis was reactive in the IgM FTA-ABS test after fractionation, and the sera of two untreated patients were nonreactive. Schmidt and Luger (19) studied 26 patients suspected, on the basis of an increasing Venereal Disease Research Laboratories (VDRL) titer, of being reinfected. Clinical signs of syphilis were present in 5 of the
patients, and 10 of the patients had sera reactive in IgM FTA-ABS test after fractionation. Wilkinson and Rodin (26) found that the number of positive IgM FTA-ABS test results for unfraccionated sera from patients with late syphilis was considerably lower than that for unfraccionated sera from patients with early syphilis. O’Neill and Nicol (15) found that all patients with late syphilis had sera that were reactive. The mechanism behind the production of IgM antibodies found in sera from reinfected patients and patients with late syphilis has not been elucidated, and the differences found in some of the studies are difficult to explain. The fixed treponemes used in the FTA-ABS test constitute a complex mosaic of antigens that may contain both T-cell-dependent and T-cell-independent antigens (e.g., polysaccharides). This may explain the IgM response in the IgM FTA-ABS test of sera from reinfected patients and patients with late syphilis. However, antibodies against purified treponeme polysaccharides in humans with syphilis have not been demonstrated (9). The IgM antibodies in reinfected persons therefore may bind to previously unidentified T-cell-independent antigens or to epitopes not detected by the immune system the first time the patient had syphilis. Alternatively, the IgM antibodies may be produced by clones of B-lymphocytes hidden during the first infection.

Six sera from patients with primary syphilis were reactive in the anti-IgM ELISA and nonreactive in the IgG ELISA, indicating that the anti-IgM ELISA has an acceptable sensitivity. However, the sera of 10 patients with primary and secondary syphilis (groups 1 and 2, respectively) were nonreactive in the anti-IgM ELISA, although they were reactive in the IgG ELISA and other syphilis serological tests. These 10 patients may have had syphilis previously if an IgM response is obligatory in the first infection. One of the patients had a sore on his penis 10 years previously that was suspected of being a chancre, but he was not treated at that time. Six of the 10 patients were foreigners and could have been treated for syphilis in their home countries without our knowledge. Of the other 139 patients in groups 1 and 2, only 13 were foreigners.

After treatment, the IgM antibodies decreased rapidly (group 10; Fig. 2). However, the sera of three patients (group 5) were reactive more than 10 months after treatment. One of these patients had been treated 33 years earlier. We do not believe that this patient had late syphilis since the sera of all patients with late syphilis (Fig. 1 [group 4]) were nonreactive. Long-time duration of the IgM response in some patients has also been shown in the IgM FTA-ABS test (15, 26).

The specificity of the anti-IgM ELISA was high: 98.6% for the patients whose sera gave false-positive reactions (group 6). In comparison, the specificity of the IgG ELISA was 93.2% ($P \leq 0.05$).

The anti-IgM ELISA might be useful for the serodiagnosis of neonatal congenital syphilis since interaction of rheumatoid factors and IgG-IgM competition were not seen (16). None of the sera from the newborns (group 9) with IgG antibodies from the mothers were reactive in the anti-IgM ELISA. Sera from children with congenital syphilis could not be investigated since this manifestation is extremely rare in Denmark, i.e., less than one case every 5 years.

In conclusion, the anti-IgM ELISA for antibodies against the flagellum may be a useful addendum to the IgG ELISA. An IgM-reactive serum indicates that the patient should receive antisyphilis treatment if he or she has not been treated recently. An IgM-nonreactive serum does not exclude the possibility that the patient needs treatment; in fact, the patient may be reinfected or have late untreated syphilis. The anti-IgM ELISA may assist in distinguishing between early latent syphilis (IgM reactive) and late latent syphilis (IgM nonreactive) and may prove useful for the diagnosis of congenital syphilis in newborns. Interference from rheumatoid factor and IgG-IgM competition are not experienced, and serum fractionation is not needed.

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