Demonstration of Immunoglobulin M Class Antibodies to Toxoplasma gondii Antigenic Component p35000 by Enzyme-Linked Antigen Immunosorbent Assay

E.-G. LINDENSCHMIDT
Institute of Medical Microbiology and Immunology, University of Hamburg, 2000 Hamburg 20,
Federal Republic of Germany

Received 21 January 1986/Accepted 10 September 1986

On the basis that 89% of 48 acute-phase toxoplasmosis patients showed immunoglobulin M (IgM) class antibodies to the 35,000-molecular-weight antigenic component (p35000) of Toxoplasma gondii, as demonstrated by IgM immunoblotting, the antigen was purified by sucrose gradient centrifugation and enzyme labeled for use in an enzyme-linked antigen immunosorbent assay (ELA) for the demonstration of IgM class antibodies to the p35000 component. The ELA showed a specificity of 96% with 139 serum specimens at a serum dilution of only 1:5. The test serologically detected 73 symptomatic acute-phase toxoplasmosis patients; 64 were positive in the 19S IgM indirect immunofluorescent-antibody test, and 9 were negative, although they showed IgM antibodies to p35000, as demonstrated by IgM immunoblotting. Also, the ELA turned out to be independent of IgM rheumatoid factors in six acute-phase toxoplasmosis serum specimens.

The demonstration of immunoglobulin M (IgM) class antibodies to Toxoplasma gondii is of clinical value in the assessment of symptomatic or asymptomatic acute toxoplasmosis (9). Standard techniques established for this purpose are the 19S IgM indirect immunofluorescent-antibody test (IIF) and the double-sandwich IgM enzyme-linked immunosorbent assay (ELISA) (11). The specificities of both tests appear to be comparable (7), and the sensitivity of the ELISA is greater than that of the 19S IgM IIF (19). However, these tests are laborious to establish and prone to disturbances due to IgM class rheumatoid factors (IgM RFs) and antinuclear factor (3, 10). In previous studies on the demonstration of specific IgM class antibodies, it was proposed that IgM RFs do not interfere with an ELISA to detect specific IgM if an enzyme-linked antigen immunosorbent assay (ELA) is used (16, 25). The studies presented here were designed to demonstrate the advantages of using an ELA to detect IgM class antibodies to the 35-kilodalton (kDa) major antigenic polypeptide component of T. gondii (IgM-p35000-ELA).

MATERIALS AND METHODS

Sera. Serum specimens were investigated from 130 patients showing lymphadenopathy and IgG antibody titers $\geq 1:4,000$ to T. gondii by IIF, which raised clinical suspicion of symptomatic acute toxoplasmosis. Also, sera of 139 healthy blood donors were used that showed various titers ($\leq 1:1,000$) of these specific IgG antibodies and no IgM class antibodies to the protozoan.

IIF. The IIF was performed to demonstrate IgG or IgM antibodies to T. gondii. Specific IgG antibodies were evaluated quantitatively, starting with a serum dilution of 1:4. Commercial lyophilized T. gondii cells (bioMérieux lot 0311) were used as antigen. Fluorescein isothiocyanate-labeled anti-human IgG reagent (Dako, Copenhagen, Denmark) from rabbits was used at a dilution of 1:80 in phosphate-buffered saline (PBS) (pH 7.4). IgM class antibodies to the protozoan were detected in the 19S IgM serum fractions obtained after gel chromatography using Ultrogel AcA34 and PBS for the elution. Columns were loaded with 700 $\mu$l of serum, and four or five 19S IgM fractions of 1.3 ml each (total $\leq 7$ ml) were collected, which indicated a less than 1:10 dilution of serum during gel chromatography. Specific IgM class antibodies complexed with membrane antigens of the commercial T. gondii cells were demonstrated with fluorescein isothiocyanate-coupled anti-human IgM reagent (Dako) diluted 1:40 in PBS.

Demonstration of IgM RFs directed to human IgG antibodies. Demonstration of IgM RFs directed to human IgG antibodies was performed by a slightly modified ELISA, as described previously (7). For this test, IgG antibodies to T. gondii were purified from serum specimens of acute-phase toxoplasmosis patients (positive for 19S IgM and also for IgG $\geq 1:256,000$ by IIF) by using ion-exchange chromatography over DEAE-Sephacel. Fractions of purified IgG to T. gondii showed titers of 1:1000 to 1:2,000, and no IgM class antibodies were detectable by immunodiffusion or by 19S IgM IIF. Dynatech microtiter plates were coated with (per cup) 5 $\mu$g of purified IgG antibodies to T. gondii diluted in isotonic bicarbonate buffer (pH 9.6; coating buffer) during an 18-h incubation at 4°C, and the antibodies were then denatured for 20 min at 74°C (18) in a moist chamber. Test sera were diluted 1:5, 1:50, 1:500, and 1:5,000 in PBS and incubated for 2 h at 37°C in a moist chamber to have antibodies captured at the solid phase which were directed to human IgG. After three washes with 0.5% Tween 20 in PBS (washing solution), 50 $\mu$l of anti-human IgM reagent (Dako) coupled with horseradish peroxidase (type V1; Sigma Chemical Co., St. Louis, Mo.) and diluted 1:500 in 0.5% Tween 20 in PBS was added to each cup. This immunoreaction at the solid phase was allowed to occur for 18 h at room temperature and was conceived to detect IgM class antibodies complexed with IgG to T. gondii via the Inv or Gm allotypes (1). After three washing steps, the enzyme reaction was performed for 5 min at room temperature in the dark using 100 $\mu$l of $o$-phenylenediamine (10 mg/10 ml of 0.1 M Na$_2$PO$_4$-0.1 M K$_2$HPO$_4$) per cup and was stopped with 100 $\mu$l of 4 M H$_2$SO$_4$. Extinctions at 492 nm were read automatically in a Multiscan (Titertek). Negative control sera were negative for RFs in the hemagglutination test of
FIG. 1. Demonstration of antigenic components of T. gondii by IgG immunoblotting (lane a) and by IgM immunoblotting for three acute-phase toxoplasmosis patients (lanes B through D). The positions of molecular size markers (M) in kilodaltons are shown on the left.

Waaler (24) and devoid of IgG and IgM antibodies to T. gondii. Extinctions exceeding the double mean value of at least eight negative controls indicated a positive ELISA for IgM antibodies to IgG antibodies directed to T. gondii.

Preparation and demonstration of T. gondii antigenic components. Preparation and demonstration of T. gondii antigenic components was done initially using tachyzoites of the RH strain cultivated in a human epithelial (HEp) cell line (23) in Eagle minimal essential medium supplemented with fetal calf serum (2.5%), gentamicin (80 mg/liter), and nystatin (4 mg diluted in 1 ml of dimethylformamide per liter). Cytopathic effect was observed 2 to 3 days after inoculation, and T. gondii cells were harvested mainly from days 4 to 12. They were separated from HEp cells by passage through a filter (Selecta 47108; pore size, 3 μm) and purified from the medium by five washes in PBS, each followed by a centrifugation at 3,000 × g for 10 min at 4°C in a Christ centrifuge. The cells were lysed first by freezing (−80°C) and thawing (4°C) three times, then by an overnight incubation in 2 mM Tris (pH 7.4) diluted in bidistilled water, and finally by six ultrasonication steps for 45 s each in an ice bath. Residual particles were sedimented (10 min at 5,000 × g). The supernatant contained soluble antigens of T. gondii at a protein concentration of 8 to 12 mg/ml. Protein estimations were done by the method of Lowry et al. (8). Soluble antigens were dialyzed against Laemmli buffer (5), and 100-μl aliquots were stored at −80°C.

Dissolved antigenic components were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis by the method of Laemmli (5) with an 11% slab gel. Electrophoresis was performed for 3 h at 25 mA, staining was done with 0.001% Coomassie blue in 50% methanol-10% acetic acid for 18 h, and destaining was done in 5% methanol-7% acetic acid for 24 h.

IgG and IgM class antibodies to different antigenic components of T. gondii were demonstrated by immunoblotting, performed as described previously (13, 17). The antigens separated by SDS-polyacrylamide gel electrophoresis were transferred electrophoretically (22) onto nitrocellulose paper at 200 mA for 14 h at 4°C in 192 mM glycine-20% (vol/vol) methanol-25 mM Tris hydrochloride (pH 8.3). Blocking was done first in 0.5% Tween 20 in PBS for 15 min and later in 10% skim milk powder (Oxoid Ltd., London, England) in 0.5% Tween 20 in PBS for 30 min. Nitrocellulose strips (2 to 3 mm) were exposed to human test sera diluted 1:50 in 0.5% Tween 20 in PBS for 18 h and, after an additional blocking procedure for 4 h, to anti-human IgG (Fig. 1, lane A) or anti-human IgM (Fig. 1, lanes B through D) reagent labeled with alkaline phosphatase (Dako; 1:1,000 in 0.5% Tween 20 in PBS). The enzyme reaction was performed with Nitro Blue Tetratolium (0.1 mg/ml) and 0.01% indolylphosphate dissolved in 1.1 M veronal in acetic acid (pH 9.6) for 3 to 5 min in the dark.

Preparation of the p35000 antigenic component of T. gondii. Protein (10 mg) from a T. gondii whole-cell lysate was adjusted to 192 mM glycine–2% mercaptoethanol–1% SDS–25 mM Tris hydrochloride (pH 8.3), boiled for 3 min, loaded on a sucrose gradient (15 to 60% in the same reducing medium), and centrifuged in an SW40 rotor in a Beckman L-50 centrifuge for 72 h at 38,000 rpm at 10°C. Later, 15 or 16 gradient fractions of about 850 μl each were collected (Fig. 2).

The fractions were investigated for antigenic components of T. gondii by an ELISA as follows. Samples of the fractions (50 μl) were diluted in coating buffer and allowed to absorb to the solid phase of Dynatech microtiter plates for 18 h at 4°C. After three washes, the cups were incubated with 100 μl of a 19S IgM fraction positive by IIF at 1:40 dilution. Specific IgM complexed with antigenic components of the protozoan at the solid phase was detected with horseradish peroxidase-labeled anti-human IgM reagent, as described for the ELISA established to demonstrate IgM RFs. The fractions showing a positive ELISA for antigenic components of T. gondii were investigated for the p35000 polypeptide by SDS-polyacrylamide gel electrophoresis and IgM immuno-
blotting (Fig. 2). Two nonprotein bands due to 2-mercaptoethanol at the 54- and 68-kDa positions were visible on the gel, as described by others (21).

Antigen labeling of the p35000 antigenic component. The fractions containing the antigenic component p35000 obtained after sucrose gradient centrifugation were dialyzed against 192 mM glycine–2% 2-mercaptoethanol–25 mM Tris hydrochloride (pH 8.4) to remove SDS. The protein was labeled with horseradish peroxidase (type VI; Sigma) by the periodate method of Wilson and Nakane (26). Labeled antigen was dissolved in Laemmli buffer without SDS but with 5% fetal calf serum and 0.001% Merthiolate and stored as 50-µl aliquots at −80°C.

ELISA for demonstration of IgM class antibodies to p35000. Dynatech microtiter plates were coated with anti-human IgM reagent (Dako) diluted 1:1,000 for 18 h at 4°C and washed three times afterward. Test serum specimens were diluted 1:5, 1:25, 1:125, 1:625, etc., in PBS and incubated for 2 h at 37°C in a moist chamber. Enzyme-labeled p35000 antigen diluted 1:1,000 in 0.5% Tween 20 in PBS (50 µl per cup) was allowed to react for 2 h at 37°C in a moist chamber. After three washing steps, the enzyme reaction was performed and interpreted as described for the IgM RF ELISA. Negative controls did not show any specific IgG or IgM class antibodies, as demonstrated by IIF.

### RESULTS

IgM class antibodies to different antigenic components of *T. gondii* during acute toxoplasmosis. The IgM immunoblots for 3 of 48 investigated acute-phase toxoplasmosis patients are shown in Fig. 1, lanes B through D. The first shows IgM class antibodies complexed with the 6-, 14-, 25-, 30-, 35-, 40-, and 50-kDa polypeptides of *T. gondii*; the second shows IgM class antibodies to only the 6-, 25-, and 35-kDa components; the third shows IgM class antibodies to the 6-kDa component and 35-kDa polypeptide.

The frequency of detection of the different IgM class antibodies directed to various *T. gondii* antigens in 48 acute-phase toxoplasmosis patients is presented in Table 1. IgM class antibodies to the 6-kDa polypeptide were seen in 97% of the patients, and IgM class antibodies to the 35-kDa component were seen in 89% of the patients.

ELISA for demonstration of IgM-RFs directed to human IgG. Of 73 acute-phase toxoplasmosis patients positive for 19S IgM to *T. gondii* by IIF, 20 (27%) showed IgM class antibodies complexed with IgG of *T. gondii* (IgM RFs), as detected by the ELISA. These antibodies were demonstrated at a titer of 1:5 in six patients (30%), 1:50 in nine patients (45%), 1:500 in three patients (15%), and ≥1:5,000 in 2 patients (10%).

**Table 1. Occurrence of IgM class antibodies to different antigenic components of *T. gondii* in 48 acute-phase toxoplasmosis patients**

<table>
<thead>
<tr>
<th>Mol wt of antigenic component</th>
<th>No. (%) of patients with IgM class antibodies to antigenic component</th>
</tr>
</thead>
<tbody>
<tr>
<td>50,000</td>
<td>33 (69)</td>
</tr>
<tr>
<td>40,000</td>
<td>39 (81)</td>
</tr>
<tr>
<td>35,000</td>
<td>43 (89)</td>
</tr>
<tr>
<td>30,000</td>
<td>15 (39)</td>
</tr>
<tr>
<td>25,000</td>
<td>12 (25)</td>
</tr>
<tr>
<td>14,000</td>
<td>3 (6)</td>
</tr>
<tr>
<td>6,000</td>
<td>47 (97)</td>
</tr>
</tbody>
</table>

**Table 2. Specificity of IgM-p35000-ELA determined with sera of 139 healthy blood donors negative for 19S IgM to *T. gondii***

<table>
<thead>
<tr>
<th>IgG IF titer</th>
<th>No. of sera tested</th>
<th>No. of sera with indicated IgM-p35000-ELA result at serum dilution:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:16</td>
<td>14</td>
<td>1 (1:25)</td>
</tr>
<tr>
<td>1:64</td>
<td>11</td>
<td>1 (1:25)</td>
</tr>
<tr>
<td>1:256</td>
<td>20</td>
<td>2 (1:25)</td>
</tr>
<tr>
<td>1:1,000</td>
<td>35</td>
<td>1 (1:25)</td>
</tr>
<tr>
<td>1:4,000</td>
<td>20</td>
<td>1 (1:25)</td>
</tr>
<tr>
<td>1:16,000</td>
<td>19</td>
<td>1 (1:25)</td>
</tr>
<tr>
<td>1:64,000</td>
<td>16</td>
<td>1 (1:25)</td>
</tr>
<tr>
<td>1:256,000</td>
<td>4</td>
<td>1 (1:25)</td>
</tr>
</tbody>
</table>

DISCUSSION

The demonstration of IgM class antibodies to *T. gondii* is urgently needed in any microbiobiologic laboratory. 19S IgM IIF requires separation of IgG and IgM antibodies and also prevention of IgM RF interference. The double-sandwich ELISA for the detection of IgM to *T. gondii* needs soluble antigens of the protozoan, purified and enzyme-labeled IgG to *T. gondii*, and control for IgM RF as well as antinuclear factor (11). IgM-p35000-ELA was easier to establish and provided us with several advantages over the double-sandwich IgM ELISA. Advantages of the ELA were also reported recently by Neurath et al. (12) in detecting antibodies to the p24 component of human T cell lymphotrophic virus type III by using enzyme-labeled core protein (p24) which they precipitated in polyethylene glycol 6000 after an extraction of the p24 antigen from the virus.

Earlier investigations revealed that the p35000 antigenic component of the *T. gondii* membrane (2, 4, 15, 17) is the

**Table 3. Accracy of 19S IgM IIF and IgM-p35000-ELA**

<table>
<thead>
<tr>
<th>Result of 19S IgM IIF</th>
<th>No. of sera at indicated dilution with IgM-p35000-ELA result:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive (≥1:25)</td>
<td>64 (8)</td>
</tr>
<tr>
<td>Negative</td>
<td>9 (49)</td>
</tr>
</tbody>
</table>

Downloaded from [http://jcm.asm.org/](http://jcm.asm.org/) on February 6, 2021 by guest
most immunogenic constituent of tachyzoites, representing 3 to 5% of the total protein of the protozoan (13). Our investigations on the demonstration of different IgM class antibodies to different antigens of T. gondii in 48 acute-phase toxoplasmosis patients by using IgM immunoblotting showed that those directed to the p35000 component were detectable in almost 90% of the patients. This was lower only than the frequency of detection of antibodies directed to the 6-kDa antigenic component (97%). The 6-kDa antigenic component did not appear suitable for ELA use since it is assumed to be a minor protein component of the protozoan, consisting mainly of carbohydrates (17), and since its purification from the 14-kDa component seems to be difficult. We succeeded in isolating the p35000 antigenic component by isopycnic sucrose gradient centrifugation, keeping the epitope stable in the reducing buffer of Laemmli et al. (5).

The ELA established for the demonstration of IgM class antibodies to the p35000 component provided us with several advantages over the standard techniques to detect IgM class antibodies to T. gondii. First, this test appears to allow specific detection of the IgM class antibodies at an initial serum dilution of only 1:5 (Table 2). This is different from the double-sandwich IgM ELISA, which should not be used at a serum dilution lower than 1:40 (6). Second, the IgM-p35000-ELA appears to be independent of specific IgG antibodies. A specificity of >95% for this test at a dilution of 1:5 at both low and high titers of IgG to T. gondii is shown in Table 2. Also, competition of high-titered IgG antibodies with specific IgM class antibodies, resulting in reduced IgM titers or false-negative IgM tests (20, 25), is not to be expected if the monospecificity of the anti-human IgM reagent at the dilution applied is guaranteed. Monospecificity was confirmed for our studies by a negative IIF for the 7S IgG fractions obtained after gel chromatography of an acute-phase toxoplasmosis serum (IgG titer, 1:4 × 10^6 by IIF), using our fluorescein isothiocyanate-labeled anti-human IgM reagent diluted 1:1,000. Third, this test was shown to be independent of IgM RFs in six cases. Fourth, it was suggested previously (14) that the demonstration of IgM class antibodies to the p35000 antigenic component of T. gondii is highly indicative of a rather early stage of infection. Therefore, a negative IgM-p35000-ELA does not completely rule out acute toxoplasmosis (Table 1) if serum samples are taken late during IgM-positive disease. Such samples need to be tested by 19S IgM IIF or IgM ELISA if clinical signs are highly indicative of acute disease. For these reasons and because of its ease of performance and the stability of the purified p35000 antigenic component in Laemmi buffer at −80°C for at least 3 months, we prefer to use the IgM-p35000-ELA to screen for early acute toxoplasmosis.

ACKNOWLEDGMENTS

The RH strain of T. gondii was kindly provided by K. Mannweiler, Director, Department of Bacteriology/Serology, Bernhard Nocht Institute, Hamburg, Federal Republic of Germany. G. Winterseel performed the standard tests skillfully. H. von Wulffen kindly read this paper critically.

LITERATURE CITED

