Toxoplasma gondii infection: analysis of serological response by 2-DE immunoblotting

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Abstract

Toxoplasma gondii is known to cause a variety of diseases ranging from asymptomatic infections to serious conditions in immunocompromised hosts such as AIDS-patients or transplant recipients. In addition they may cause abortion or fetal abnormalities during pregnancy. Despite the clinical importance, diagnosis, treatment and prevention still remain unsatisfactory. Analysis of the parasitic cell determinants, recognized by specific humoral and cellular immune responses, may have important implications for diagnosis, therapy and vaccination strategies. Two-dimensional electrophoresis (2-DE) was used to resolve and compare protein patterns from Toxoplasma gondii strains RH and BK (mouse virulent strains). Comparison of silver-stained gels showed that 35.2% to 60.3% of the spots had the same position. In a second series of experiments, the reactivity of the spots with human sera was tested. Proteins were transferred to PVDF membranes and were detected with sera from different patient groups. Depending upon the immunoglobulin class (IgG, IgM, IgA or IgE) different epitope patterns were observed. Some of the spots seemed to be recognized in different stages of infection. Sera of two patients with similar serology and comparable stage of infection were compared in order to demonstrate an individual immune response. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Toxoplasma gondii; 2-Dimensional electrophoresis; Serology; Immunoblot

1. Introduction

Toxoplasmosis is one of the most prevalent parasitic infections in Europe. On a global scale approximately 30% of the world population carries this parasite. The causative organism Toxoplasma gondii exists in two forms in humans, the actively proliferating trophozoites (or tachyzoites) and the slower growing bradyzoites [1]. Parasites attach via parasite-ligand cell receptor interaction and penetrate the host cell membrane and multiply by endodyogeny [2]. Proliferation slows down under the influence of immune responses finally resulting in cyst formation. These cysts are infective if ingested by a new host. The final host for these parasites are felines. In the feline intestine T. gondii trophozoites produce both macro- and micro-gametes [3], which form sporocysts after fecundation. These are shed in the
environment and may infect a variety of intermediate hosts, including man. Phylogenetic and statistical analysis of several isolates indicated a highly unusual population structure consisting of three widespread clonal lineages [4], which were shown to be antigenically different [4–6]. Regardless of the host or geographic origin, the virulent strains of *T. gondii* comprise a single lineage [7], which is characterized by a 23 kDa membrane antigen that is used to classify the virulent character [8].

In immunocompetent hosts infections with *T. gondii* are generally mild or asymptomatic. However, if acquired first during pregnancy, parasites may cross the placenta and infect the fetus. During pregnancy, it is essential to assess the time of infection. While the probability of transmission increases with time and is highest at the end of pregnancy, the consequences of infection are more severe during organogenesis in the early phases of pregnancy. Infections in later stages of pregnancy result in persistent fetal infections with a high risk of late complications such as retinchorioiditis in the newborn in about half of all acute maternal infections. It is therefore essential to adequately treat women during pregnancy, to reduce the frequency of fetal infections and the severity of sequelae.

Up to 90% of primary infections in pregnant women are not diagnosed [9]. At present, the detection is exclusively based on serological screening and molecular methods (PCR). However, the use of serology alone is insufficient to assess the risk of the active disease, especially during pregnancy and in immunocompromised patients as reactivation of a latent toxoplasmosis occurs frequently in these patients.

In AIDS-patients, *T. gondii* is the main reason for often lethal intracerebral lesions. Early detection of the disease is therefore required to initiate effective therapy.

To improve the diagnostic we started the separation of antigens of *T. gondii* hoping to screen for an antigen which reacts in a specific manner with antibodies from different immunoglobulin classes during the acute phase of infection. For this purpose we divided the patients sera in our study into three groups with respect to the clinical course or duration of the infection. In each case the specific reaction of the immunoglobulin classes was determined.

To get a high resolution of antigens, we used the resolving power of two-dimensional electrophoresis (2-DE), combining isoelectric focusing in the first dimension and SDS-PAGE in the second. Using PDQUEST software we were able to separate about 300 protein spots in *T. gondii* whole cell homogenates.

Some spots (3, 7, 9, 10, 13, 16, 21, 32 and 35) reacted with all classes of immunoglobulins. There was no difference with regard to the state of infection. Nevertheless seven other spots (1, 2, 15, 18, 19, 24 and 31) showed some special features concerning their reaction pattern. These may help to improve serodiagnoses.

### 2. Material and methods

#### 2.1. Toxoplasma strains, culture and sample preparation

*T. gondii* RH and BK strains (both kindly provided by Dr. Janitschke, Robert Koch Institute Berlin, Germany) were maintained by serial passages in the human amnion cell line FL521 (kindly provided by Prof. Krüger, Institute of Virology, Charité Berlin, Germany) in a HEPES-buffered RPMI-1640 medium (Seromed, Germany) supplemented with 10% fetal calf serum (FCS Boehringer Mannheim, Germany) containing 100 μg ml⁻¹ streptomycin and penicillin, respectively (Gibco BRL, Paisley, UK). For large scale cultures 600 cm³ plastic culture flasks (Greiner GmbH, Germany) were used. The *T. gondii* cells were washed twice in 0.05 M Tris/HCl (Merck, Germany) and centrifuged at 11 000 g for 2 to 5 min (Biofuge 28RS Heraeus separatech, Germany, rotor number 3745). The pellets were frozen and thawed twice in a lysis buffer (Klose and Kobalz, 1996 [10]), and sonicated at 50 kHz per 20 s (Bandelin Sonopuls GM 70/UW 70, Germany). The parasite lysate was prepared for 2-DE by adding a solution containing 9 M urea (Bio-Rad, Germany), 10% dithiothreitol (DTT, Serva, Germany) and 10% Servalyte 2–4 (Serva, Germany). The protein concentration was determined using a Coomassie Blue Protein Assay Reagent (Pierce, USA).

Aliquots of a protein solution containing 4.5 mg protein ml⁻¹ were applied.
The apparent molecular mass (MM) and the isoelectric point (pI) were determined by coelectrophoresis of standard solution of marker proteins (Bio-Rad, Germany).

2.2. 2-Dimensional electrophoresis

2-DE was performed using the method described by Jungblut and Seifert [11]. The separation gel consisted of 9 M urea (Bio-Rad, Germany), 5% (w/v) glycerol (Merck, Germany), 3.5% acrylamide (Bio-Rad, Germany), 0.3% N,N-methylene-bis-acrylamide (BIS Bio-Rad, Germany), 0.06% (v/v) N,N,N,N-tetramethylethylenediamine (TEMED Bio-Rad, Germany), 1.33% (w/v) Servalyte pH 5–7 (Pharmacia, Sweden), 0.67% Ampholytes pH 3–10 (Pharmacia, Sweden) and 0.02% (w/v) ammoniumpersulfate (AP, Bio-Rad, Germany). The running conditions were as follows: cathode solution: 9 M urea (Bio-Rad, Germany), 5% (w/v) glycerol (ICN Biomedicals, Ohio, USA) and 5% (v/v) ethylenediamine (Merck, Germany); anode solution: 3 M urea (Bio-Rad, Germany) and 745 mM phosphoric acid (Merck, Germany). The proteins were focussed for 75 min at 100 V, 200 V, 400 V and 600 V, for 10 min at 800 V, and for 5 min at 1000 V. Subsequently gels were incubated in a solution with 125 mM Tris-phosphate (pH 6.9 Merck) 40% glycerol (ICN Biomedicals, Ohio, USA) and 3% sodiumdodecylsulfate (SDS, Bio-Rad, Germany) for 5 min and frozen if not processed immediately.

SDS-PAGE was performed with modifications according to Laemmli [12], using Mini-Protean II cells (Bio-Rad, Germany). The separation gel consisted of 0.375 M Tris-HCl, 0.03% TEMED, 15% acrylamide, 0.2% BIS, 0.1% SDS and 0.08% AP. The rod gels (first dimension) were applied onto the slab gels in 1% agarose (Serva, Germany). The running conditions were: 5 min at 35 V, 10 min at 55 V, 15 min at 100 V and 1 h at 150 V, using a Phero-stab 300 powersupply (Biotec-Fischer, Germany). Before staining gels were incubated in a fixing solution (50% ethanol, Merck, Germany and 10% acetic acid, Merck, Germany) overnight. For immunoblotting, the gels were processed unfixed.

2.3. Silver-staining

Silver-staining was performed as described by Heukeshoven and Dernick [13] and modified as described by Jungblut and Seifert [11]. The stained gels were dried using the ANAMED drying kit (NOVEX, USA) according to the manufacturer’s recommendations.

2.4. Immunoblotting

Proteins were transferred using standard blotting techniques and a Bio-Rad Germany electrotransfer chamber. The blotting buffer contained 39 mM glycine (ICN Biomedicals Ohio, USA), 48 mM Tris and 20% methanol (J.T. Becker, The Netherlands).

To obtain identical replicas from one gel proteins were transferred to Immobilon P membranes (Millipore, USA) for 8 h at 250 mA using the double replica blotting technque as described by Johannson [14]. The immunoblots were developed according to Towbin et al. [15].

Membranes were blocked overnight with BSA (3%; Sigma, USA) in PBS saline buffer (pH 7.4) and incubated with patients sera in dilutions ranging from 1:50 to 1:150 for 1 h by room temperature (RT). After washing, membranes were incubated with alkaline phosphatase or peroxidase conjugated goat anti-human IgG, IgM, IgA and IgE sera (1:250; Bio-Rad, Germany) in PBS saline buffer with 0.05% Tween-20. Finally the appropriate substrate NBT/BCIP (50 mg ml⁻¹)(Bio-Rad, Germany) was added. This procedure was done according to the manufacturer’s recommendations.

2.5. Monoclonal antibodies

Some spots were identified by monoclonal antibodies, which were kindly provided by Dr. Windeck, Magdeburg, Germany. Antibodies used: antibody 5G9 (subtype IgG1): a cytosol protein specific antibody; antibody 1H5 (subtype IgG2a): a cytosol specific antibody; and antibody SAG1/P30 (GII-9) and IgG (subtype IgG2a): recognizes a 30 kDa tachyzoite respectively 37 kDa membrane antigens.
2.6. Patients sera

Twenty-one sera from 11 pregnant women with T. gondii infections at different stages as defined by classical serological methods (Vidas Toxo-IgG, Vidas Toxo-IgM bio-Merieux Sa, France and Platelia Toxo-IgA Sanofi-Pasteur, France) were used (group 1). The sera were routinely obtained from individual patients at different times. In parallel sera and/or CSF (cerebrospinal fluid) from newborns, fetal cord blood samples and amniotic fluids were collected. Eight sera from six patients with acute toxoplasmosis (group 2) and nine sera from nine healthy, latently infected persons as shown by their IgG titer (group 3) were also analyzed. Two sera of healthy volunteers served as controls. All patients sera were obtained during routine diagnostics.

2.7. Scanning and spot detection

The PDQUEST® system (PDI, USA) was used for the computer-assisted image analysis of gels and membranes. The scanner converted the original silver-stained gels into a digitized image. Horizontal and vertical streaks and background noise were suppressed. Spot detection yielding an image for each protein spot was fitted by a two-dimensional Gaussian curve. All spots were matched to detect qualitative and quantitative differences between the gels.

3. Results

The small gel technique (7×9 cm) of 2-DE showed reproducible protein patterns with reasonable resolution. Fig. 1 shows a typical, silver-stained gel of T. gondii RH strain. Proteins in the range of 15–90 kDa and pI 5.0–8.4 were separated. Data of those spots that most frequently reacted with all sera used are listed in Table 1 and demonstrated in Fig. 2. A total of 281 ± 23 or 311 ± 7 protein spots were counted in the RH an BK antigen preparations respectively (see Fig. 3).

The number of matching protein spots in prepara-

![Fig. 1. 2-DE protein pattern of Toxoplasma gondii strain RH: small gel technique. The original gel size was 8.5 cm×7.5 cm×1.5 mm. The pattern was calibrated according to molecular masses (Mr) and apparent pI (pl) by marker proteins (see Section 2). The gels were scanned with HP-ScanJet 3c.](image-url)
tions of *T. gondii* strains (RH and BK) was in the range of about 35–60% when comparing gels run at different runs.

The degree of similarity ranged from $r = 0.53$ to $r = 0.75$, with a mean of $r = 0.60$ as calculated by PDQUEST software. Correlation coefficient '$r'$ is a measure of the quantitative similarity between gels.

Some spots were identified by monoclonal antibodies (see Fig. 2). The antibody 5G9 reacted with a spot group in a range $pI < 5$ and MM 34 kDa (spot number 24) and antibody 1H5 also recognized a group of spots (e.g. spot number 3: 36.0 kDa, $pI$ 7.7–8.4). A 30 kDa tachyzoite membrane antigen reacted with the antibody SAG1/P30 (GII-9). The membrane specific monoclonal antibody 1G8 reacted with a few spots (e.g. spot number 6: 31.3 kDa and $pI$ 7.0).

Fig. 4 shows a representative Western blot of 2-DE separated *T. gondii* proteins using antibodies specific for the four immunoglobulin classes. We concentrated our attention on 42 frequently reacting spots, which were well distributed with regard to their molecular masses and isoelectric points.

Some spots reacted with all classes of immunoglobulins (3, 7, 9, 10, 16, 21, 32 and 35), which were of low interest with regard to discrimination of stage of infection.

Seven spots showed a special picture concerning their reaction patterns. The data obtained with regard to the reaction of the different immunoglobulin classes comparing the three groups of patients are given in Table 2. Spot 1 gave a strong positive reaction in all three patients groups only with IgG. In addition there was a reaction with IgM, which was strongest in group 2. There was no reaction of IgA and IgE antibodies of the latent infected group (group 3). Spot 2 gave positive reactions in all groups with IgG. There was no reaction with IgM, IgA and IgE in the latent infected group (group 3), and only the acute infected group 2 reacted with IgE.

Spot 15 reacted with all antibody classes in all three groups of sera, but the reaction in the group with latent infected persons was much more frequent. Spot 18 gave a positive reaction with IgG and IgE in all groups, the reaction with IgE in the latent group was especially frequent. A positive re-

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**Table 1**

Molecular mass and isoelectric point of frequently reacting protein spots with patients sera

<table>
<thead>
<tr>
<th>Spot number</th>
<th>MM</th>
<th>$pI$</th>
<th>Spot number</th>
<th>MM</th>
<th>$pI$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>32.5</td>
<td>7.4</td>
<td>22</td>
<td>65.0</td>
<td>5.6–6.65</td>
</tr>
<tr>
<td>2</td>
<td>33.1</td>
<td>&lt;5.0</td>
<td>23</td>
<td>34.0</td>
<td>6.9</td>
</tr>
<tr>
<td>3</td>
<td>36.0</td>
<td>7.7–&gt;8.4</td>
<td>24</td>
<td>34.0</td>
<td>&lt;5.0</td>
</tr>
<tr>
<td>4</td>
<td>36.5</td>
<td>6.7</td>
<td>25</td>
<td>34.0</td>
<td>5.9</td>
</tr>
<tr>
<td>5</td>
<td>36.4</td>
<td>5.9</td>
<td>26</td>
<td>50.4</td>
<td>5.15</td>
</tr>
<tr>
<td>6</td>
<td>31.3</td>
<td>7.0</td>
<td>27</td>
<td>44.2</td>
<td>5.15</td>
</tr>
<tr>
<td>7</td>
<td>45.4–45.5</td>
<td>6.3–7.0</td>
<td>28</td>
<td>48.2</td>
<td>5.2</td>
</tr>
<tr>
<td>8</td>
<td>55.6</td>
<td>5.6</td>
<td>29</td>
<td>31.0</td>
<td>8.4</td>
</tr>
<tr>
<td>9</td>
<td>61.7</td>
<td>5.1</td>
<td>30</td>
<td>48.0</td>
<td>5.6</td>
</tr>
<tr>
<td>10</td>
<td>41.0</td>
<td>7.5–8.4</td>
<td>31</td>
<td>17.3</td>
<td>8.4</td>
</tr>
<tr>
<td>11</td>
<td>22.5</td>
<td>6.0</td>
<td>32</td>
<td>52.0</td>
<td>8.4</td>
</tr>
<tr>
<td>12</td>
<td>62.5</td>
<td>7.0</td>
<td>33</td>
<td>34.3</td>
<td>5.8</td>
</tr>
<tr>
<td>13</td>
<td>37.1</td>
<td>5.7</td>
<td>34</td>
<td>38.9</td>
<td>7.0</td>
</tr>
<tr>
<td>14</td>
<td>34.0</td>
<td>5.8</td>
<td>35</td>
<td>50.7</td>
<td>8.4</td>
</tr>
<tr>
<td>15</td>
<td>57.1</td>
<td>5.7</td>
<td>36</td>
<td>24.0</td>
<td>8.1</td>
</tr>
<tr>
<td>16</td>
<td>44.1</td>
<td>5.8</td>
<td>37</td>
<td>33.0</td>
<td>5.6</td>
</tr>
<tr>
<td>17</td>
<td>44.1</td>
<td>5.6</td>
<td>38</td>
<td>25.4</td>
<td>7.3</td>
</tr>
<tr>
<td>18</td>
<td>73.0</td>
<td>5.7</td>
<td>39</td>
<td>18.2</td>
<td>5.0</td>
</tr>
<tr>
<td>19</td>
<td>32.4</td>
<td>6.4</td>
<td>40</td>
<td>43.0</td>
<td>5.4</td>
</tr>
<tr>
<td>20</td>
<td>33.0</td>
<td>8.4</td>
<td>41</td>
<td>83.0</td>
<td>5.1</td>
</tr>
<tr>
<td>21</td>
<td>37.5</td>
<td>7.4–8.4</td>
<td>42</td>
<td>30.0</td>
<td>8.4</td>
</tr>
</tbody>
</table>

For estimation of molecular mass (MM) and isoelectric point ($pI$) of proteins, seven marker with known MM and $pI$ were coelectrophoresed. Protein standard used were from Bio-Rad Germany.
action with IgM and IgA appeared only in the groups with acute infections. Spot 19 gave a strong or frequent positive reaction with IgG in all groups. Positive reactions with IgM and IgA, as in the case of spot 18, appeared only in the group with acute infection. Also there was a positive reaction with IgE more frequently in the group with latent infected persons. However there was no reaction in group 2. Spot 24 reacted with IgG antibodies in all groups. There was a positive reaction with IgM, IgA and IgE only in the group with acute toxoplasmosis, which was most frequent in the acute group 2. Spot 31 gave positive reactions with IgE only in the group with acute infections and more pronouncedly in group 2 than in group 1. In case of IgA the reaction was more frequent in groups 1 and 2 as expected.

Antibodies present in the other materials, like the amniotic fluid, the cord blood sample and the cerebrospinal fluid (CSF), reacted with the same spots [3, 7, 9, 10, 13, 16, 21, 25, 32, 35] which were most frequently detected by all four immunoglobulin classes present in the patients sera. Only a few particularities were observed: Spot 1 gave positive responses exclusively with IgG. Spot 2 reacted with IgG in cord blood sample and amniotic fluid. Spot 15 reacted with IgA and IgM in cord blood sample. Spot 18 gave an intensively positive reaction with IgG in cord blood samples and amniotic fluid. Spot 19 reacted with IgA and IgM in CSF. Spot 24 and spot 31 reacted often with all immunoglobulin classes. The number of experiments is too small to see significant differences. Therefore, these data are not shown in detail.

To obtain information about the variation between different patients, the complete data obtained
Table 2
Reactions of antibodies of different immunoglobulin classes of sera from three patients groups (group 1, pregnant women with acute toxoplasmosis; group 2, patients with acute toxoplasmosis; group 3, patients with latent toxoplasmosis) with seven spots; the sera were used in a dilution of 1:100

<table>
<thead>
<tr>
<th>negative reaction</th>
<th>% of positive reactions</th>
</tr>
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<tr>
<td></td>
<td>&lt;10%</td>
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<tr>
<td></td>
<td>10-20%</td>
</tr>
<tr>
<td></td>
<td>20-40%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>patients:</th>
<th>1. group</th>
<th>2. group</th>
<th>3. group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
<td>IgM</td>
<td>IgE</td>
</tr>
<tr>
<td>Spotnumber</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
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<td></td>
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<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>18</td>
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<tr>
<td>19</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>31</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
with the sera of two pregnant women were compared. The serum of the first woman (L.A.: 29 years old, seroconversion in the 11th week of pregnancy, therapy with sulfadiazine, pyrimethamine and folinic acid, fetal outcome unknown, because of change of medical care but sonography in the 24th week of pregnancy revealed no fetal defects) had an IgG value of 99 IU ml\(^{-1}\), an IgM quotient > 20 and an IgA quotient of 8.6. The anti-Toxoplasma antibody titer determined by immunofluorescence (IFT) was 1:4000. The serum of the second woman (W.A.: 26 years old, seroconversion in the 36th week of pregnancy, therapy with sulfadiazine, pyrimethamine and folinic acid, infection of the fetus was probable, two cerebral foci were found) had a serum value of IgG 103 IU ml\(^{-1}\) an IgM quotient > 20 and an IgA quotient of 6.2. The IFT titer was also 1:4000. The anamnesis of both women relating to social status, childhood diseases and allergic situation were similar.

The reacting spots (number and position) are shown in Table 3. The unique spots are proteins, which reacted only with the serum of one patient. The remaining spots reacted with both sera. Although the serological status of both women was similar, their qualitative results, testing reactivity with 2-DE protein spots from \textit{T. gondii} were different.

4. Discussion

Our aim was to find specific antigen markers for more precise serodiagnosis. Using 2-DE we were able to detect 281 ± 23 or 311 ± 7 protein spots of BK and RH strains of \textit{T. gondii} respectively. To describe the differences in the protein pattern of these two \textit{T. gondii} strains, a ‘subtractive approach’ was chosen. Comparing all gels of one species with each gel of the other species we found differences...
between the protein pattern of RH and BK strain, of 39.7% to 64.8% depending on preparation, with a mean value of 51%.

We have two explanations for these differences: There may be a varying degree of modification resulting in differing protein groups and there are differences in gene expression depending on slightly varying growth conditions which result in quantitative differences.

Although the antigen composition of *T. gondii* was extensively investigated in the past, using this new type of approach one has the possibility to identify more proteins and to get more information about known antigens. The use of two-dimensional electrophoresis led Kasper [17] to the conclusion that the known P30 protein is composed of more than one polypeptide.

Only few groups investigated *T. gondii* by means of 2-DE. Segers [18] separated several hundred spots, but in the immunoblot he only found 20–50 spots that reacted with IgG. No other immunoglobulin classes were investigated. All data available show that only a small part of the proteins will induce antibody production during normal infection. Handman [19] detected and characterized several membrane antigens. She found four major antigens using monoclonal antibodies. The mAbs 2G11 (IgG2) and 3E6 (IgG2) she used, reacted with antigens of 35 and 14 kDa molecular mass respectively. The mAb 1E3 (IgG2) detected a protein of 43 kDa and the mAb 1E11 (IgG3) a protein of 27 kDa. Her study was the first characterization of membrane antigens of *T. gondii*. Using the immunoblot technique she also showed great variability of the reaction with human sera [20]. Her comparison of three strains of *T. gondii* RH, C37 and C56 revealed no differences between strains. However she used one-dimensional SDS-PAGE.

Because the data in the literature concerning 2-DE of *T. gondii* are limited, it was difficult to compare our data with other described antigens of *T. gondii*, which were mainly received by Western blotting; this means in one dimension only. We found a lot of spots and spot groups with a range of molecular masses comparable to the known proteins e.g., P22, P23, P30, P35, P37, P39, P42, P45, P73 and P83, which were described by Ware [21], Huskinson [22] and Moir [23]. Further characterization has to be done, to prove the identity.

There is still controversy about possible differences between BK and RH strains in human infections. Although these two strains are members of the same clonal lineage, *T. gondii* strain RH belongs to genotype I and is extremely virulent in mice, causing significant higher levels of parasitaemia [16]. Both strains are used to produce antigens for conventional serodiagnostics.

In our study there were 42 protein spots which reacted with most sera and with antibodies of different classes. These are given in Table 1. There were

<table>
<thead>
<tr>
<th>Immunoglobulins</th>
<th>Patient L.A.</th>
<th>Patient W.A.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>25 protein spots (global)</td>
<td>13 protein spots (global)</td>
</tr>
<tr>
<td>Unique spots (spot number)</td>
<td>15 (3, 4, 6, 7, 9, 11, 12, 14, 15, 17, 24, 26, 31, 32, 35)</td>
<td>3 (19, 29, 37)</td>
</tr>
<tr>
<td>IgM</td>
<td>6 protein spots (global)</td>
<td>7 protein spots (global)</td>
</tr>
<tr>
<td>Unique spots (spot number)</td>
<td>2 (1, 13)</td>
<td>3 (20, 32, 35)</td>
</tr>
<tr>
<td>IgA</td>
<td>13 protein spots (global)</td>
<td>14 protein spots (global)</td>
</tr>
<tr>
<td>Unique spots (spot number)</td>
<td>3 (2, 11, 28)</td>
<td>4 (1, 13, 23, 25)</td>
</tr>
<tr>
<td>IgE</td>
<td>16 protein spots (global)</td>
<td>15 protein spots (global)</td>
</tr>
<tr>
<td>Unique spots (spot number)</td>
<td>8 (4, 5, 12, 20, 29, 32, 38, 42)</td>
<td>7 (13, 17, 27, 28, 31, 33, 34)</td>
</tr>
</tbody>
</table>

Patient L.A.: 29 years old, seroconversion 11th week of pregnancy; IgG 99 IU ml⁻¹, IgM positive (> 20), IgA positive (8.6).
Patient W.A.: 26 years old, seroconversion 36th week of pregnancy; IgG 103 IU ml⁻¹, IgM positive (> 20), IgA positive (6.2).

The unique spots are proteins which reacted only with antibodies serum of one patient. The difference between the protein spots (global) and unique spots number score spots which reacted with both patient sera.
no protein spots which exclusively reacted with only one immunoglobulin class, but seven spots revealed some special features with regard to stage of infection and immunoglobulin class reacting with. These results with these spots (see Table 2) will be discussed further, starting with IgG.

There were no obvious differences in the reaction profile. Three of these protein spots (number 1, 2 and 19) strongly reacted with IgG independently of the stage of infection. Verhofstede [24] found no correlation between titer and the number of bands in his study of immunoblotting with infection stage-related IgG response to T. gondii. His results indicate that the consecutive appearance of IgG antibodies during the course of infection is highly comparable for all patients studied. A 35 kDa antigen elicited the first IgG response soon after exposure. Two of our frequently reacting spots (spot 3 (MM 36.0 kDa, pI 7.7–8.4) and spot 25 (MM 34.3 kDa, pI 5.9)) have a corresponding molecular mass. Small differences in molecular mass may be explained by differences in methods used and by variations in antigen preparations.

It is of special interest that Verhofstede also found natural antibodies in negative control sera, whereas this phenomenon did not appear in our investigations.

We have no explanation, why spot 31 gave no reaction with IgG of acute toxoplasmosis patients.

In the case of IgM, protein spots 2, 18, 19, and 24 reacted more frequently with IgM antibodies in patients with acute toxoplasmosis, whereas spot 15 often reacted in latent infected patients and spots 1 and 31 showed no or only slight differences in the reactions between acute and latent infections.

Spot 15 is therefore of some interest for a discrimination between acute and latent infection.

In the literature exist very divergent observations. Bessieres [25] mentioned difficulties in diagnostic discrimination between acute and chronic diseases because of long persistent IgM antibodies. Potasman reported on naturally occurring antibodies against T. gondii [26]. Konishi [27] constantly found high IgM levels in combination with negative IgG titers, which he interpreted as naturally occurring IgM antibodies in the Japanese population.

Decoster et al. showed in 1988 [28] that in acute infections IgM antibodies against 97 different antigens seemed to be good markers of early acute infection. The comparative study of antibody responses to membrane antigens showed that in chronic toxoplasmosis human sera recognized four antigens with molecular masses of 43, 35, 30 and 22 kDa respectively, and in acute toxoplasmosis the antibodies recognized the 43 and 30 kDa antigens first. The serological profile of the newborn was different from that of his mother, with an additional antibody response to a 170 kDa antigen [28].

In our study there was a promising difference in the reaction of IgM class antibodies with spots 2, 18, 19 and 24 in dependence of the stage of infection: no reaction in latent cases.

IgA occurs 2 weeks after IgM and persist for 6 to 7 months, but there is a great variability in duration and expression [29]. A positive value for IgA antibodies gives no proof of acute toxoplasmosis in the last 3 to 6 months, but it is helpful towards understanding the development of the infection [30]. Decoster declares that screening for IgA antibodies is necessary and suggestive [31]. Saathoff found that some serum samples from patients infected for up to a year or longer were still IgA positive [32]. IgA antibodies corresponding to a variety of antigens were present in sera from patients with acute and chronic infections irrespective of the titer in an IgA ELISA.

Our data show that there are antigens that react early in infection with IgA (spots 1, 2, 18, 19 and 24) and others which also and even stronger react late (spot 15).

Naser [33] pointed out that the diagnostic value of IgE antibodies is ambiguous with respect to T. gondii infections.

In our study, there is a positive reaction with the IgE of acute infected persons in spots 1, 2, 24 and 31 without any reaction with IgE antibodies from chronically infected patients. On the other hand there is a reaction (spot 19) or even a pronounced reaction (spots 15, 18) with IgE antibodies from the chronically infected group. Wong [34] detected no IgE antibodies in seronegative persons (except one child, which was declared as false positive result). However, he found such IgE antibodies in pregnant women with seroconversion during pregnancy, in patients with toxoplasmonic lymphadenopathy and infants with congenital toxoplasmosis as in infants
and adults with toxoplasmic chorioretinitis and also in AIDS-patients with toxoplasmic encephalitis by comparing two diagnostic methods. Often he found only low IgE titers, that means a low IgE titer does not exclude an acute toxoplasmosis. In addition, even a negative result does not exclude acute infection, because there exist IgE nonresponder. Pinon [35] used an immunocapture assay to detect specific IgE antibodies in patients with toxoplasmosis. IgE antibodies appeared simultaneously with IgA and IgM antibodies but they did not persist longer than 4 months.

Using ELISA Bessieres-Cathala [36] found positive reactions of specific IgE antibodies in patients with chronic toxoplasmosis. We too found in our patients investigated by 2-DE immunoblotting a similar tendency. It was remarkable that only latent infected patients reacted fully positive with spot 15 antigen.

The evaluation of specific antibodies in other materials (amniotic fluid, cord blood, CSF) supported the data obtained with serum (data not shown). We found positive responses to protein spots 1 and 2 only with antibodies from the IgG class. These antibodies were probably originated in the mother. It was interesting to find that protein spot 15 reacted with specific antibodies of IgA and IgM classes from cord blood. These antibodies were actively produced from the fetus or newborn, respectively.

As the investigations progressed it became clear that there were big individual differences with regard to antigen recognition (Table 3). The two young women showed varying reactions with our separated antigens, although their serological status was similar (see Table 2). On the other hand, we found an almost identical reactive pattern through a comparison of four sera obtained from one patient at different stages of her infection. The differences in the reactive patterns of these two serologically comparable young women express the complexity of the immune response. The diversity could be further increased by the fact that there is a great antigen diversity among different strains of *T. gondii* [7].

The virulent strains of *T. gondii* originated from a single lineage. They are clearly different from the avirulent strains. In most diagnostic laboratories RH and BK strains, which belong to the mice virulent group, are used as the antigen source. However, most *T. gondii* strains isolated from patients belong to the mice avirulent group. There is an overwhelming evidence that the immunological status of the host plays a major part in the clinical outcome of this opportunistic infection in humans. Therefore, the patient’s specific reaction is also of great importance for diagnosis.

From the data of different immunoglobulin classes, one can conclude that analyzing different antigen spots may lead to a more specific diagnostic procedure. Spots 1, 2 and 24 using IgM, IgA and IgE and in case of spot 31 using IgE, could be markers for acute infection. This becomes even more obvious if one argues that group 2 are the really acute infected patients whereas the group 1 patients were found in a screening of pregnant women, thus including older infections too. The proposed discriminatory protein spots showed greater differences between groups 2 and 3 with group 1 more intermediate, which one would expect under these conditions.

Antibodies against spot 15 (all classes) should be an indicator of latent infections. Spot 18 can be used as a marker for acute or latent infections, depending on the immunoglobulin class detected. Spot 31 seems to be the most interesting one, because it reacts in a very specific manner with the different immunoglobulin classes. While there was no IgG reaction in the acute phase, there were frequent IgA and IgE reactions but no reaction with IgE in the latent group (see Table 2).

The seven antigens presented are candidates for a diagnostic marker group which should be accomplished by further markers. In addition, it became clear that there probably would not be a single antigen which could be used as a marker to differentiate between acute and chronic or latent infection.

This leads to the suggestion that a panel of markers, each with a given value, could be used for diagnosis comparable with the diagnostics of lyme borreliosis. To verify this hypothesis further experiments have to be carried out.

Taking this into account, we believe that it is possible to use 2-DE gels of one given *Toxoplasma* species for evaluating antibody responses even varying itself.
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