Seroreactivity to and Avidity for Recombinant Antigens in Toxoplasmosis

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Received 3 January 2005/Returned for modification 16 February 2005/Accepted 31 May 2005

To improve serodiagnostic methods for the diagnosis of acute toxoplasmosis during pregnancy, a new test system has been developed and evaluated based on the use of recombinant antigens. Five recombinant Toxoplasma gondii antigens (ROP1, MAG1, SAG1, GRA7, and GRA8) were cloned in Escherichia coli, purified, and applied directly onto nitrocellulose membranes in a line assay (recomLine Toxoplasma). A panel of 102 sera from 25 pregnant women with supposed recent toxoplasmosis and from two asymptomatic, the diagnosis is generally based on serological and applied directly onto nitrocellulose membranes in a line assay (recomLine Toxoplasma). A panel of 102 sera from 25 pregnant women with supposed recent toxoplasmosis and from two asymptomatic, the diagnosis is generally based on serological

Toxoplasmosis is caused by the parasite Toxoplasma gondii. Cats are the primary host of this pathogen. Sexual reproduction takes place only in the primary host, leading to excretion of infectious oocytes that can be incorporated by humans while gardening or by the consumption of insufficiently washed fruits or vegetables. In an alternative pathway, humans can be infected by nonparasitic material and that vary due to different antigen preparation methods are utilized.

Thus, recombinantly produced Toxoplasma gondii antigens were considered to replace tachyzoite material in toxoplasmosis serology. In the past, a large number of different recombinant antigens were produced in Escherichia coli and studied for their potential to serve as diagnostic markers of Toxoplasma gondii infections; these included dense granule proteins GRA1 (p24 [1, 2, 5]), GRA2 (p28 [25]), GRA4 (p41 [1, 20]), GRA6 (p32 [1, 26]), GRA7 (p29 [1, 2, 10, 12]), and GRA8 (p35 [1, 2, 13, 31]), surface antigens SAG1 (p30 [1, 2, 4, 7]) and SAG2 (p22 [1, 22]), rhoptry antigens ROP1 (p66 [1, 12]) and ROP2 (p54 [1, 33]), microneme proteins MFC3 and MFC5 (2); and other recombinant antigens of Toxoplasma gondii. In most studies the recombinant antigens were coated alone or in some cases in various combinations on enzyme-linked immunosorbent assay (ELISA) plates. A combination of GRA7, GRA8, and ROP1 was previously suggested for the detection of Toxoplasma gondii-specific immunoglobulin M (IgM) antibodies (1). A combination of GRA7, GRA8, and SAG1 (1) or of GRA7, GRA8, SAG2, and H4 (14) was proposed for the detection of IgG antibodies. The latter combination was supposed to be reactive in patients with acute profiles but not in patients with chronic profiles. So far, combinations of different recombinantly produced antigens have not been applied separately on nitrocellulose membranes. Such a technique would allow the detection of human anti-Toxoplasma gondii antibodies directed against every single
The main objective of all diagnostic efforts in toxoplasmosis serology (mostly as preventive measures during pregnancy) is clarification of whether or not the pregnant woman has been acutely infected or whether infection occurred before conception. Due to the fact that low IgM titers in most cases persist long beyond the acute phases of infection, confirmation of IgM antibodies in serum is an inadequate criterion for diagnosing an acute toxoplasmosis (17). Therefore, determination of the avidities of the IgG serum antibodies is a very important step in diagnostics (8, 18). However, it has been shown that the IgG antibodies raised against the individual *Toxoplasma* antigens differ in their maturation characteristics (19, 34). In particular, there were antigens found that did not induce the synthesis of high-avidity IgG antibodies at all (19, 34). Thus, using the complete mixture of tachyzoite antigens as it is used in conventional avidity assays, the determination of avidity is compromised by these differences. The use of recombinant antigens in avidity determination might overcome these limitations. This should be especially effective if the avidities of the IgG antibodies directed against the individual recombinant antigens are determined independently from one another. Applying this approach, an improved estimation of the probable time point of infection should be possible.

The aim of this study was to investigate the changes in seroreactivity to and avidity for individual recombinant antigens during the time course of infection and to examine whether differences might be exploited as diagnostic tools in toxoplasmosis serology.

**MATERIALS AND METHODS**

Cloning and expression of recombinant proteins. Genomic DNA of *Toxoplasma gondii* strain RH was isolated using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). *Toxoplasma gondii* tachyzoites (RH strain) were provided by R. Disko (Klinikum rechts der Isar, Munich, Germany). Transformation of *Escherichia coli* and the production of competent cells were carried out according to the method of Hanahan (6). Restriction endonucleases and T4 DNA ligase (Roche Diagnostics, Mannheim, Germany) were used as recommended by the manufacturer. Five immunodominant *Toxoplasma gondii* antigens, ROP1 (p66 [12]), MAG1 (p65 [12]), SAG1 (p30 [7]), GRA7 (p29 [10]), and GRA8 (p35 [12, 13]), GenBank accession numbers M71274, U09029, X14080, Y13863, and AF310261, respectively, were expressed as full-length proteins in *Escherichia coli*. The genes were amplified from *Toxoplasma gondii* by PCR with specific primers based on the sequence information obtained from the GenBank database. Useful restriction enzyme sites were incorporated into these primers. All genes were expressed without the sequences coding for the signal peptides. The PCR was carried out using a commercially available PCR kit (Roche Diagnostics). Samples were denatured at 94°C for 2 min, annealed at 45°C for 2 min, and extended at 72°C for 4 min. The total number of cycles was 30. The reaction products were analyzed by electrophoresis on 1.0% agarose gels containing ethidium bromide (0.5 mg/ml). DNA was extracted with a gel extraction kit (QIAGEN, Hilden, Germany). Subsequently, the DNA was cleaved with suitable restriction enzymes. Fragments were ligated into pUC8 or pDS1 plasmid vectors. *Escherichia coli* JM109, M15, or OmpT/H11002 strains were transformed. The cloned sequences were verified by sequence analysis. The recombinant antigens were expressed after induction with 2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 37°C. The bacteria were harvested 3 h after induction. Bacterial lysis and purification of recombinant antigens were performed following the method of Soutschek et al. (30).

Production and processing of nitrocellulose strips. Individual dilutions of the purified recombinant antigens were applied directly onto nitrocellulose membranes in different lines using a specialized device (Biodot). The appropriate line conditions for all recombinant antigens were determined empirically with standard serum samples. Membranes were blocked with 1% skim milk solution in phosphate-buffered saline, air dried, and cut into individual test strips. Strips were stored at 4°C. Processing of nitrocellulose test strips was performed according to the instruction manual for recomLine Toxoplasma (MIKROGEN) using the reagents supplied in the kit. Details are as follows. Serum samples were

FIG. 1. Reactivities of recombinant antigens to IgG (G), IgM (M), and IgA (A) antibodies and avidities of IgG antibodies (Av) in infection time course of case 1.
applied at 1:100 dilutions and incubated together with the nitrocellulose test strips for 1 h at room temperature. Following three washing steps of 5 min each, a second incubation of 45 min with peroxidase-labeled secondary antibody (anti-human IgG, IgM, or IgA) was performed. Strips were stained for about 8 min using tetramethylbenzidine after three additional washing steps of 5 min each. The reactivities of serum antibodies against the recombinant antigens were evaluated by comparison to a cutoff band. The cutoff band gives a slight and strictly reproducible reactivity with every single serum sample and can therefore be used as a reference band. Reactivities stronger than or equivalent to that of the reference band were regarded as positive, while weaker reactivities or the absence of reaction were regarded as negative. For avidity determination, strips were washed once after serum incubation and then incubated with avidity reagent for exactly 3 minutes. The protocol was continued with three washing steps. Avidity was estimated by comparison of two individual test strips, one of which was incubated with the avidity reagent. Avidity was regarded to be low if the band intensity loss significantly exceeded 50 percent. Avidity was regarded to be intermediate if the band intensity loss was about 50 percent. Avidity was regarded to be high if band intensity loss was significantly less than 50 percent.

Sera. All serum samples were analyzed and characterized by Cobas Core IgG (Roche) and by ETI-TOXOK-M reverse PLUS and partly by ETI-TOXOK-A reverse PLUS (DiaSorin). In addition, IgG avidities were determined using VIDAS TOXO IgG AVIDITY (BioMerieux). According to the manufacturer, high-avidity test results are present only in individuals who have been infected for at least 4 months. All test systems were applied according to the manufacturers’ instructions. Serum samples were collected from patients suspected of having acute toxoplasmosis (panel A: IgM positive, IgG positive or IgG seroconversion on second sample, and/or IgG low avidity) and from individuals with chronic toxoplasmosis (panel B: IgG positive and IgM negative, with IgG avidity not tested). Panel A consisted of 102 sera from 27 patients (25 pregnant woman and two children) collected at two to seven samplings ranging from 4 weeks to 6 months from the first to the last sample tested. This panel was divided into three subgroups. Subgroup A1 included all serum samples of panel A collected initially in the course of infection. Subgroup A2 comprised the subsequent sera taken until 3 months after the first serum sample. Subgroup A3 comprised sera taken subsequently between 3 and 6 months after the first serum sample. Panel B consisted of 71 sera of 71 patients.

RESULTS

We cloned and expressed five recombinant antigens, ROP1 (p66), MAG1 (p65), SAG1 (p30), GRA7 (p29), and GRA8 (p35), of *Toxoplasma gondii* in *Escherichia coli*. The antigens were selected from a larger panel of antigens investigated (including in addition GRA4, GRA6, ROP2, and SAG4) and proved to be most suitable for serodiagnosis (data not shown). The recombinant antigens were purified by standard chromatographic methods and applied directly onto nitrocellulose for a line assay. These test strips (Fig. 1 and 2) were used to investigate different serum panels for IgG, IgM, and IgA antibody patterns and IgG avidity.

Comparison of antibody patterns in acute and past infections. Antibody results with the recomLine Toxoplasma assay are presented in Tables 1 and 2. IgM and IgA activities with the recombinants showed essentially no distinctive features over time. However, IgG reactivities with MAG1, SAG1, and GRA8 showed different levels over times of infection. Examples of antibody reactivity are shown in Fig. 1 and 2. In initial specimens from six patients, the recombinant line assay detected *Toxoplasma*-specific IgG antibodies which were not detected by the Cobas Core IgG.

Development of the immune response in individual time courses of infection. To further demonstrate the typical fea-
tures of the immune response development over time directed against the individual recombinant antigens, two representa-
tive cases from panel A will be discussed in detail.

The serum samples of case 1 were derived from a lymphad-
enopathy patient who received no therapeutic treatment. In
contrast, all pregnant women included in this study received
therapeutic treatment either by spiramycin or by a combina-
tion of pyrithemamine and sulfadiazine. Case 1 represents a
13-year-old child suffering from lymphadenopathy. Serum
samples were collected 1, 3, and 21 months after the first onset
of symptoms. The results of the recombinant line assay are
shown in Fig. 1. In the first serum sample, IgG antibodies were
reactive to GRA7, GRA8, and ROP1, while in the second and
third samples, MAG1 and SAG1 reactions were also seen. In
the first sample, IgG antibodies against GRA8 were of high
avidity, but those directed against GRA7 and ROP1 were of
low avidity. IgG antibodies against GRA7 had already matured
by the time that sample 2 was taken, whereas antibodies di-
rected against MAG1 and SAG1 exhibited high avidities in the
last sample only. In contrast, ROP1 and GRA8 showed low-
avidity IgG antibodies in the last sample. ROP1 and GRA8
were strongly reactive to IgM antibodies in the first sample.
IgM antibody reaction diminished in the second sample and
was negative in the last sample. The IgA response was re-
stricted to ROP1 and GRA8 in the first sample and to GRA8
in the second sample.

In case 2, three serum samples were collected from a 35-
year-old woman pregnant in the last trimester (Fig. 2). Therapy
started 3 days after the collection of the first serum sample.
Analyzing the first sample in the recombinant line assay, low-
 started 3 days after the collection of the first serum sample.

### TABLE 1. Reactivities of individual recombinant antigens to
Toxoplasma gondii-specific IgG antibodies of the IgG-positive sera of
subgroups A1, A2, and A3 and to all sera of panel B

| Recombinant antigen | Subgroup A1 | Subgroup A2 | Subgroup A3 | Panel B |
|---------------------|-------------|-------------|-------------|---------|
|                     | (n = 22)    | (n = 31)    | (n = 10)    | (n = 71) |
| ROP1                | 50.0 (11)   | 52.9 (27)   | 40.0 (4)    | 38.0 (27) |
| MAG1                | 31.8 (7)    | 56.9 (29)   | 70.0 (7)    | 62.0 (44) |
| SAG1                | 9.1 (2)     | 29.4 (15)   | 60.0 (6)    | 91.5 (65) |
| GRA7                | 90.9 (20)   | 94.1 (48)   | 90.0 (9)    | 88.7 (63) |
| GRA8                | 95.5 (21)   | 94.1 (48)   | 100.0 (10)  | 46.5 (33) |

### TABLE 2. Reactivities of individual recombinant antigens to
Toxoplasma gondii-specific IgM and IgA antibodies of
panel A and panel B

| Recombinant antigen | Panel A (n = 102) | Panel B (n = 71) |
|---------------------|-------------------|-----------------|
|                     | positive for:     | positive for:   |
|                     | IgM (n = 68)      | IgA (n = 34)    | IgM | IgA |
| ROP1                | 92.6 (63)         | 32.3 (11)       | 7.0 (5)* | 0.0 (0) |
| MAG1                | 11.8 (8)          | 8.8 (3)         | 0.0 (0) | 0.0 (0) |
| SAG1                | 7.4 (5)           | 2.9 (1)         | 0.0 (0) | 0.0 (0) |
| GRA7                | 20.6 (14)         | 47.1 (16)       | 0.0 (0) | 0.0 (0) |
| GRA8                | 64.7 (44)         | 67.6 (23)       | 0.0 (0) | 1.4 (1) |

* All these samples had very high levels of IgG (ELISA value > 1.5; cutoff value, 0.58) and strongly reacted to IgG with all five recombinant antigens.

ROP1 were detected, but no antibodies directed against
MAG1 or SAG1 were detected. IgM (ROP1, MAG1, GRA8)
and IgA antibody reactions (GRA8) were positive. Three
weeks later, IgG avidity to GRA7 had already increased, while
IgG antibodies against MAG1 appeared. IgM antibody reac-
tions were unchanged (only the reaction with MAG1 was
weaker), and IgA antibody reactions decreased. In the last
serum sample, collected 7 months after the first one, IgG
antibodies of high avidity for ROP1, MAG1, SAG1, and
GRA7 were accompanied by antibodies of low avidity for
GRA8. Neither IgM nor IgA antibodies against the recombi-

### DISCUSSION

So far, many different recombinant antigens have been pro-
duced and characterized for their abilities to detect Toxo-
plasma gondii-specific antibodies. In general, only the reactiv-
ities of single recombinant antigens were investigated, or
cocktails of recombinant antigens were produced and analyzed
in combination with the ELISA technique (1, 14, 24). Up to the
time of this study, differentiation between individual reactivi-
ties of a combined panel of recombinant antigens was not
examined. These differentiations as well as the avidities of
human Toxoplasma gondii-specific IgG antibodies directed
against single antigens have been evaluated so far using only
Toxoplasma gondii lysates analyzed by Western blotting (19,
34).

In this work, we evaluated the individual reactivities and IgG
avidities of five different Toxoplasma gondii antigens cloned
and expressed in Escherichia coli, which were combined for a
line assay. We tested the IgG, IgM, and IgA reactivities to and
IgG avidities of Toxoplasma gondii-specific human sera for
these recombinant antigens, using two different panels of hu-
man sera which were characteristic for presumed acute and
chronic infections.

For patients with presumed acute toxoplasmosis, positive
IgM results in the recombinant line assay were provoked
mainly by IgM antibodies directed against ROP1 and to a
lesser extent GRA8. The importance of these antigens in IgM
serology relative to toxoplasmosis has already been described
(1, 12). No information on the actual state of infection could
be concluded from the IgM antibody pattern. ROP1 was respon-
sible for the long-term persistence of the IgM in the recombi-
nant line assay in most cases. This might explain why ROP1
reacted with IgM of some strongly IgG-positive sera in the
group of samples that were IgM negative by ELISA. Never-
theless, unspecific reactivity of ROP1 in those cases cannot be
excluded.

The IgA responses in the recombinant line assay varied
greatly, an observation that is consistent with the results of
earlier studies (9). Quite often, response was lacking com-
pletely. The most prominent antigens reactive with human IgA
antibodies were GRA8 and GRA7. The IgA pattern itself gave
no substantial information on the actual state of infection.

In contrast to findings with IgM as well as with IgA, the IgG
response showed characteristic patterns that could be ex-
ploited to distinguish acute from chronic infections. Even in
the very early phase of infection, the recombinant antigens
reacted with IgG antibodies, whereas the reference test system

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was still negative. This finding suggests a higher sensitivity of the recombinant line assay with these sera. At the beginning of the IgG response, IgG antibodies to GRA7 and/or GRA8 were exclusively present, whereas IgG antibodies to MAG1 and/or SAG1 were still lacking. In all time courses of infection, IgG antibodies to MAG1 and, in particular, to SAG1 were observed significantly later. In addition, the IgG antibodies against MAG1 and SAG1 matured at significantly later time points than those directed against GRA7 and GRA8. These findings can easily be observed in both time courses of infection shown as examples (Fig. 1 and Fig. 2) and were observed in all cases of this study. In previous studies, it has been also shown that the maturation characteristics of the IgG antibodies against individual antigens are substantially different (3, 19, 34), but so far no attempt has been made to take advantage of these findings in order to improve the serodiagnosis of toxoplasmosis.

ROP1 and to a lesser extent GRA8 failed to trigger the maturation of IgG antibodies to high avidity in every single case, whereas IgG antibodies directed against GRA7, MAG1, and SAG1 matured in all observed cases to high avidity. Thus, the analysis of the IgG avidity for individual recombinant antigens might enable a more accurate diagnosis of acute toxoplasmosis than that of an IgG avidity determination applying tachyzoite lysates, as has already been suggested by Beghetto et al. (3). Whereas the work of Beghetto et al. (3) focuses on single recombinant antigen, MIC3, for avidity determination, our study includes five recombinant antigens that show different characteristics with regard to avidity maturation. This should be suitable to give a more differentiated picture of the avidity maturation process.

In cases of infections acquired long ago, IgG antibodies against SAG1 and GRA7 mainly dominated over IgG antibodies against the other recombinant antigens investigated, particularly over ROP1 and GRA8, in this study. The latter observation is consistent with previous findings (13). Nevertheless, GRA8 cannot be utilized as a marker of acute infections, because IgG antibodies to GRA8 persist far beyond the acute phase of infection. Whereas in most studies SAG1 was found to induce IgG antibodies as soon as the acute phase of infection (4, 24), our study shows no reactivity of IgG antibodies against SAG1 in the early stages of infection. Our study findings are supported by another study (1). These eminently different findings in the levels of recognition of recombinant SAG1 by human sera might be the result of variations in the preparations of this very complex molecule that were applied in the different studies. Thus, different epitopes might be presented dependent on the preparation technique.

The benefit of analyzing the human immune response against different recombinant antigens individually for characterization of infections caused by *Toxoplasma gondii* could be demonstrated. The combination of IgG reactivity to and IgG avidity for five individual antigens in the recombinant line assay can be regarded as a valuable tool to distinguish acute from chronic toxoplasmosis in pregnancy using a single serum sample. Nevertheless, further and more extensive studies are required and will be performed to strengthen and specify the findings presented in this study.

ACKNOWLEDGMENTS

We acknowledge Silvia Dorn and Veronika Rilling for helpful discussions, Alexandra Prepper for critical reading of the manuscript, and Manfred Motz for support.

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