Use of MAG1 Recombinant Antigen for Diagnosis of Toxoplasma gondii Infection in Humans

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This paper describes the cloning, purification, and serological applications of matrix antigen MAG1 of Toxoplasma gondii. The expression system used allows the production of a large amount of T. gondii recombinant protein, which was assessed for its potential use in an enzyme-linked immunosorbent assay (ELISA) for detection of T. gondii infection in humans. Serum samples from 117 patients with different stages of infection, along with 10 serum samples from seronegative patients obtained for routine diagnostic tests, were used. The results were compared with those of an ELISA that uses a native T. gondii antigen extract. The MAG1 antigen detected antibodies more frequently from the acute stage (97.3%) than from the chronic stage (7.5%) of toxoplasmosis. Hence, this antigen may be used as a tool for detection of T. gondii immunoglobulin G antibodies in persons with acute toxoplasmosis.

Toxoplasma gondii (a member of the phylum Apicomplexa) is an ubiquitous protozoan parasite that infects a broad range of hosts, including humans and domestic animals. There are three infectious stages in the life cycle of T. gondii, i.e., the tachyzoite stage; the bradyzoite stage, which occurs in tissue cysts; and the sporozoite stage, which occurs in sporulated oocysts (4). Tachyzoites are involved in propagation within a host, and the other two forms are involved in transmission to new hosts. However, although the ultrastructures of the three stages are similar, there are distinct differences in the phenotypes in the host and in the expression of specific proteins. Several authors have described specific molecular markers associated with both tachyzoites and bradyzoites of T. gondii (7, 10). A 65-kDa protein, MAG1, was originally described as being expressed specifically during bradyzoite development because it was localized to the ground substance of the tissue cyst and could be detected in immunoblots of extracts from cysts but not from tachyzoites (21). In 2002, Ferguson and Parmley (6) showed that MAG1 is expressed during both tachyzoite and bradyzoite development and is not a bradyzoite-specific protein. The MAG1 antigen is a very immunogenic protein. High titers of immunoglobulin G (IgG) antibodies against MAG1 are induced in infected humans and pigs (3, 9). Several authors have described the protective effects of MAG1 immunization, as a recombinant protein or as DNA vaccines, in mouse models (19, 20). Taken together, these results suggest that matrix antigen MAG1 is particularly promising as a tool for the serodiagnosis of toxoplasmosis in humans.

There are two major situations in which the diagnosis of T. gondii infection is of medical importance: first, to detect the transmission of parasites via the placenta from an infected mother to the fetus, and second, to detect the reactivation of a chronic infection in immunocompromised patients. Among the available commercial diagnostic tests, serology is commonly used. The specificities and sensitivities of these serological methods and the differentiation between the phases of toxoplasmosis depend mostly on the diagnostic antigen(s) used. At present, the detection of specific antibodies based on the recognition of crude Toxoplasma antigens requires mass production of the parasite either from the peritoneal fluids of infected mice or from tissue cultures. Recombinant antigenic proteins would be alternative sources of antigens. An advantage would be the reduced test costs due to the lower costs of production and purification of recombinant antigens. Furthermore, properly selected recombinant antigenic proteins (specific molecular markers) would detect all serologically positive individuals, as well as differentiate between acute and chronic infections.

In the present study, we have evaluated the usefulness of the MAG1 T. gondii recombinant antigen in diagnostic tests. Our results suggest that the MAG1 protein may be useful for detection of the early phase of infection with T. gondii when it is used in an enzyme-linked immunosorbent assay (ELISA) and a Western blot analysis.

MATERIALS AND METHODS

Construction of expression plasmid. The Escherichia coli TOP10F' strain (Invitrogen, Carlsbad, CA) was used for preparation of the plasmid and for cloning, and the E. coli Rosetta(DE3)(pLysS) strain (Promega, Madison, WI) was applied to express the recombinant antigen. pUET1 (DNA-Gdańsk II s.c., Gdańsk, Poland) was used for construction of the expression system. The E. coli cells with the plasmids were cultured acrobically at 37°C in LB medium supplemented with 12.5 μg/ml tetracycline and 100 μg/ml ampicillin for the E. coli TOP10F' strain and with 34 μg/ml chloramphenicol and 100 μg/ml ampicillin for the E. coli Rosetta(DE3)(pLysS) strain. Restriction enzymes were purchased from New England BioLabs. The reagents for PCR were obtained from DNA-Gdańsk II s.c. Ni²⁺-iminodiacetic acid-Sepharose was obtained from Novagen. Isopropyl-β-D-thiogalactopyranoside, agarose, and all reagents for protein purification were purchased from Sigma.

The nucleotide sequence of the T. gondii gene encoding the MAG1 antigen was obtained from the GenBank database (accession number AF251813). Tachyzoites from the T. gondii RH strain were used to isolate genomic DNA. This was used as the template for amplification of the antigen by a standard PCR amplification protocol with the following primers: 5'-GAA GTA GAT CTG
AGC CAA AGG GTG CCA GAG CTA CC-3 (forward) and 5'-CAC CCC AAG CCTT ACC AGA TCC CTG AAC CCT TAG-3' (reverse). The primers contained the BglII and HindIII recognition sequences (underlined) to facilitate cloning. The PCR product was digested with both BglII and HindIII and inserted into the BglII and HindIII sites of the pUET vector. The resulting plasmid, pUET-MAG1, contained a truncated sequence of MAG1 (from 30 to 222 amino acid residues) which was embedded in frame between His-tag domains. The nucleotide sequence of the insert was verified by the dideoxy termination sequencing method.

Expression and purification of recombinant MAG1 protein. The E. coli Rosetta(DE3)(pLysS) strain transformed with pUET-MAG1 or pUET1 was grown overnight with vigorous shaking in LB medium supplemented with 34 μg/ml of chloramphenicol and 100 μg/ml of ampicillin at room temperature. Next, 1,000 ml of LB medium supplemented with the same antibiotics was inoculated with 20 ml of this culture. The culture was grown with vigorous shaking at room temperature to an optical density at 600 nm of 0.4. Protein production was then induced with isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM, and the cells were incubated with vigorous shaking for an additional 16 h. The cells were harvested by centrifugation, and the pellet was resuspended in 20 ml of buffer A (20 mM Tris-HCl [pH 7.9], 500 mM NaCl, 5 mM imidazole, 0.1% Triton X-100, 5 M urea). The cells were disrupted by sonication, and insoluble debris was removed by centrifugation. Then the supernatant (about 20 ml) was loaded onto an Ni²⁺-imidotriacetic acid-Sepharose column (bed volume, 20 ml; Novagen) preequilibrated with 4 volumes of buffer A. After the column was loaded, it was washed twice with 20 ml of the same buffer until the UV absorption returned to the baseline. After the column was washed six times with buffer B (buffer A containing 20 mM imidazole), recombinant MAG1 was eluted with 20 ml of elution buffer, buffer C (20 mM Tris-HCl [pH 7.9], 500 mM NaCl, 500 mM imidazole, 0.1% Triton X-100). The pooled fraction containing the recombinant MAG1 was dialyzed against a phosphate-buffered saline (PBS) buffer (1% [wt/vol] NaCl, 0.075% [wt/vol] KCl, 0.14% [wt/vol] NaH₂PO₄, and 0.0125% [wt/vol] KH₂PO₄).

Electrophoresis and Western blot analysis. The protein fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12% polyacrylamide gel stained with Coomassie blue. The amount of recombinant protein was evaluated by the Bradford assay with bovine serum albumin as the standard. Western blot analysis was performed as described previously (21).

Native T. gondii antigen extract. Antigens from tachyzoites (RH strain) were prepared by the method used by Pietkiewicz et al. (25).

Recombinant ELISA. MaxiSorp multiwell plates (Nunc, Denmark) were coated with the MAG1 recombinant protein at a concentration of 1 μg/ml in a coating buffer (50 mM NaHCO₃, buffer, pH 9.6) or with a native antigen of T. gondii diluted 1:100 in the same buffer. Control wells for pooled sera were coated with a protein sample of E. coli Rosetta(DE3)(pLysS) transformed with pUET1 obtained by the same purification method and with the same dilution used for the recombinant T. gondii antigen. After overnight incubation at 4°C, the plates were washed three times with PBS–Triton X-100 and blocked at 37°C for 1 h with blocking solution (5% bovine serum albumin and 1% Triton X-100 in PBS). Subsequently, they were incubated for 1 h at 37°C with human serum diluted 1:100 in the blocking solution. The plates were then washed three times with PBS–Triton X-100 and anti-human IgG peroxidase-labeled conjugates (1 mg/ml; Sigma), diluted 1:100 in blocking solutions, were added to each well. The plates were incubated for 30 min at 37°C and then washed. Finally, enzymatic activity was revealed by incubating the plates with the chromogenic substrate o-phenylenediamine dihydrochloride (Sigma). After 45 min at 37°C in the dark, the color development reaction was stopped by adding 0.1 ml of 1 M sulfuric acid and the color intensity was measured in a microtiter plate reader (Organon-Teknika) at 492 nm.

Each serum sample was examined twice, and the results were determined for each serum sample by calculating the mean value of the optical density (OD) reading for duplicate wells. A positive result was estimated as any value higher than the average OD reading plus 3 standard deviations (cutoff) obtained with 10 serum samples from the negative control group. The cutoff values were as follows: 0.27 for rMAG1 and 0.22 for the native antigen. An ELISA performed with the control E. coli Rosetta(DE3)(pLysS)(pUET1) antigen showed an OD consistently below 0.18 (data not shown), which showed that possible contamination of the recombinant antigen with E. coli antigens did not influence the recombinant ELISA results.

Human serum samples. All serum samples used in this study were received from among samples submitted for routine screening for toxoplasmosis. A total of 127 human serum samples were analyzed and divided into three groups on the basis of serological profiles previously characterized by conventional laboratory assays that made it possible to classify the samples as follows.

Group I consisted of 37 human serum samples from patients with an acute phase of toxoplasmosis. The presence of specific IgM antibodies was measured by IgM-ELISA Vidas (bioMérieux, France) and Toxo-ISAGA plus IgMIA tests. All serum samples were positive for IgM antibodies (IgG-ELISA Vidas, bioMérieux, France); 33 serum samples had low avidities for IgG and 4 serum samples had borderline avidities for IgG, as determined by a commercial antibody avidity test (Vidas Toxo-IgG avidity; bioMérieux); and lacked specific IgM antibodies.

Group II included 80 human serum samples from patients who were found to have infections that had been acquired in the distant past (chronic toxoplasmosis). All these serum samples were positive for IgG antibodies (IgG-ELISA Vidas; bioMérieux); had high avidities for IgG, as determined by a commercial antibody avidity test (Vidas Toxo-IgG avidity; bioMérieux); and lacked specific IgM antibodies.

Group III included 10 human serum samples from T. gondii-negative nonreactive patients (the control group, which was negative for anti-T. gondii antibodies).

Statistical analysis. Statistical analysis of the ELISA results was performed with the Microsoft Excel 2003 program for evaluation of the χ² test results. Differences were considered significant when P was equal to 0.0001 (for the recombinant fusion MAG1 [rMAG1]).

Two-dimensional immunoblotting. Separation of native T. gondii antigen extracts (from the virulent RH strain and the avirulent SSI 119 strain) by two-dimensional electrophoresis, followed by immunoblotting, was performed as described by Nicken et al. (18).

Immunoblot detection of the MAG1 protein was performed with polyclonal rabbit monospecific anti-rMAG1 antibodies. The separated proteins were transferred to a nitrocellulose membrane for 2.5 h at 450 mA by using a Hoefer TE 77 semidry transfer unit. The membrane was blocked for 16 h with 3% serum albumin in PBS at 4°C; washed three times with washing buffer (0.1 M Tris base, 0.17 M NaCl, and 0.05% [vol/vol] Tween 20); and incubated for 4 h at room temperature, followed by incubation for 16 h at 4°C with anti-rMAG1 serum diluted 1:100 in the wash buffer. Next, the membrane was washed three times, incubated with anti-rabbit alkaline phosphatase-labeled conjugate (Dako, Hamburg, Germany) for 1 h at room temperature, and developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate for 7 min.

RESULTS

Expression, purification, and reactivity of the recombinant MAG1 antigen. The recombinant pUET-MAG1 construct retained the open reading frame encoding amino acid residues from 30 to 222 of the MAG1 protein and a cluster of six histidine residues for purification of the recombinant protein by metal affinity chromatography at the N and C termini. rMAG1 was expressed as a soluble protein with a calculated molecular mass of 23.8 kDa (222 amino acid residues).

The rMAG1 protein was purified by a one-step chromatography procedure by metal affinity chromatography with Ni²⁺ bound to iminodiacetic acid-agarose (Novagen). The overexpression system applied produces about 90 mg purified rMAG1 protein from 1 liter of induced culture. The purification resulted in an electrophoretically homogeneous preparation of rMAG1 (approximately 97% purity; results not shown).

The immunoreactivity of the purified antigen was tested by Western blot analysis with 10 human serum samples chosen from each of the three groups of sera included in the study. Sera from patients with an acute phase of toxoplasmosis (positive for IgM and for IgG at a low avidity) reacted strongly with the rMAG1 antigen (Fig. 1A), whereas the immunoreactivity of the rMAG1 antigen with serum samples from patients with chronic toxoplasmosis (negative for IgM and positive for IgG at a high avidity) was low (Fig. 1B). Immunoreactivity of the rMAG1 antigen with serum samples from T. gondii-negative nonreactive patients (the control group) was not observed (Fig. 1C).
Reactivity of human sera with rMAG1 fusion protein (rMAG1-ELISA) and *T. gondii* native antigen (nat-ELISA). A total of 37 serum samples from group I (with an acute phase of toxoplasmosis), 80 serum samples from group II (with chronic toxoplasmosis), and serum samples from 10 healthy individuals (group III; negative controls) were examined separately by ELISA with the rMAG1 protein (rMAG1-ELISA) or the *T. gondii* native antigen (nat-ELISA) in parallel. Sera from group III were used to obtain the relative absorbance of each serum sample and the cutoff value. The sensitivity of the rMAG1-ELISA for all serum samples tested in this study (groups I and II, from patient with acute and chronic stages of infection, respectively) was considerably lower (35.9%) than that of the nat-ELISA (89.7%). However, sera from persons suspected of having acute toxoplasmosis with low or borderline avidity (group I) reacted with the rMAG1 antigen with a high sensitivity (97.3%; 36 of 37 serum samples), whereas a much lower sensitivity (7.5%; 6 of 80 serum samples) was obtained with sera from patients with chronic toxoplasmosis with a high avidity (group II) (Fig. 2A). Statistical analysis confirmed the high sensitivity of the rMAG1-ELISA for sera from patients with acute toxoplasmosis, and these results were statistically significant ($\chi^2 = 83.091; P = 0.0001$).

The sensitivity of the nat-ELISA (97.3%) was identical to that of the rMAG1-ELISA with sera from group I but was significantly higher (86.25%; 69 of 80 serum samples) with sera from group II (Fig. 2B). Analysis of these results detected no statistically significant differences ($\chi^2 = 3.075; P > 0.079$).

All sera from the control group III (IgG negative) were negative by both the rMAG1-ELISA and the nat-ELISA.

Two-dimensional immunoblotting revealed MAG1 expression in tachyzoites. rMAG1 was identified in tachyzoites by two-dimensional electrophoresis, followed by immunoblotting detection with polyclonal rabbit monospecific anti-rMAG1 antibodies (Fig. 3). This was observed with both the virulent strain (strain RH) and the avirulent strain (strain SSI 119) of *T. gondii*. In addition, the MAG1 antigen was found as a double band, which could represent two different stages of protein processing.

**DISCUSSION**

Recombinantly produced *T. gondii* antigens were considered replacements for tachyzoite material in toxoplasmosis serology. In the past, a large number of different recombinant antigens have been produced in *E. coli* and studied for their potential to serve as diagnostic markers of *T. gondii* infections. This approach would allow detection of human anti-*T. gondii* antibodies directed against every single recombinant antigen and determination of the phase of toxoplasmosis.

In this study, we used the efficient expression system with *E. coli*, developed in our laboratory, and a simple purification method described previously (12, 13) to obtain a new recombinant MAG1 antigen of *T. gondii*. The expression system used allows the production of a large amount of immunologically active *T. gondii* recombinant protein. The large-scale purification of the fusion product yielded approximately 90 mg of rMAG1 protein per liter of culture. The efficiency of this method of rMAG1 production is much higher than that of the glutathione S-transferase fusion MAG1 protein production method previously obtained by Di Cristina et al. (3).

The main objective of all diagnostic efforts in toxoplasmosis serology (mostly as preventative measures during pregnancy) is to clarify whether or not a pregnant woman has been acutely infected or whether the infection occurred before conception. Because in most cases low IgM titers persist far beyond the acute phases of infection, confirmation of the presence of IgM antibodies in serum is an inadequate criterion for the diagnosis of acute toxoplasmosis (14). Therefore, determination of the avidities of the IgG serum antibodies is a very important step in diagnostics (11, 15). However, it has been shown that the IgG antibodies that have been raised against the individual *Toxoplasma* antigen differ in their maturation characteristics (17, 24). In particular, some antigens do not induce the synthesis of high-avidity IgG antibodies at all (17, 24). The use of recombinant antigens might overcome these limitations. In this work, we evaluated the reactivity of the MAG1 *T. gondii* antigen cloned and expressed in *E. coli*. The matrix antigen MAG1 is a protein of 65 kDa and is abundantly expressed within the...
cyst and in the cyst wall surrounding the bradyzoites. We tested the reactivity of MAG1 using two different groups of human sera that were characteristic for presumed acute and chronic infections, respectively. Both groups were analyzed by Western blotting and ELISA techniques. It was revealed that sera from patients with an acute phase of toxoplasmosis (group I; sera positive for IgM and for IgG at a low avidity) reacted much more strongly with the rMAG1 antigen than sera from patients with a chronic phase of toxoplasmosis (group II; sera negative for IgM and positive for IgG at a high avidity). This was further confirmed by the ELISA technique. The results indicate that the rMAG1-ELISA is effective in differentiating sera from patients with an acute phase of toxoplasmosis from those with a past infection (chronic toxoplasmosis). The results obtained were surprising, as MAG1 was originally described as being expressed specifically during bradyzoite development because it was localized to the ground substance of the tissue cyst and could be detected in immunoblots of extracts from cysts but those not from tachyzoites (21). Furthermore, the rMAG1 protein was recently used in a new recombinant line assay (recomLine Toxoplasma; Microgen, Germany) demonstrated by Pfrepper et al. (22). In their study, the sensitivities of a new test for the detection of IgG MAG1 antibodies in sera from individuals with acute infection were 31.8% (7 of 22 serum samples collected initially at the time of infection), 56.9% (29 of 50 serum samples taken up to 3 months after the time of retrieval of the first serum sample), and 70.0% (7 of 10 serum samples taken between 3 and 6 months after the time of retrieval of the first serum sample). Our study revealed a much higher sensitivity (97.3%) for the detection of IgG-specific MAG1 antibodies in sera from individuals with acute infection. The difference is probably due to the recombinant MAG1 that was applied. The fragment of rMAG1 described by Pfrepper et al. (22) was significantly longer (30 to 452 amino acid residues) than the rMAG1 used in this study (30 to 222 amino acid residues).

FIG. 2. Results of IgG rMAG1-ELISA (A) and IgG nat-ELISA (B) with sera from seropositive patients. Gray columns, ODs at 492 nm for 37 serum samples from patients with a recently acquired T. gondii infection (group I); clear columns, results for 80 human serum samples from patients with chronic T. gondii infections (group II); heavy horizontal line, the cutoff values (the mean plus 3 standard deviations) of 0.27 for the rMAG1-ELISA (A) and 0.22 for the nat-ELISA (B) obtained from the results obtained with sera from group III. The mean absorbance values for MAG1 were 1.056 ± 0.58 for acute-phase sera and 0.193 ± 0.08 for chronic-phase sera. The mean absorbance values for Toxoplasma lysate antigen were 0.944 ± 0.30 for acute-phase sera and 0.499 ± 0.24 for chronic-phase sera.
Earlier studies have shown that the MAG1 gene is transcription in tachyzoites and bradyzoites, suggesting that posttranscriptional regulation occurs between the developmental stages (1). This has led to the continued listing of MAG1 as a bradyzoite-specific protein (16). However, Ferguson and Parmley (6) demonstrated the presence of MAG1 protein in tachyzoites, although with a lower level of expression compared with that in cysts. In our experiment, rabbit antiserum specific for the rMAG1 antigen also reacted with a 65-kDa native antigen from tachyzoite lysates of T. gondii. The expression of MAG1 by both tachyzoites and bradyzoites has been also confirmed by microarray analysis (2).

Di Cristina et al. (3) revealed that the immunoreactivities of two distinct fragments of the BAG1 and MAG1 genes, expressed as glutathione S-transferase fusion products, could be assessed with the antibodies of T. gondii-seropositive donors. IgG antibodies from 85% of infected individuals reacted with the BAG1 and/or the MAG1 antigen fragments, emphasizing the broad recognition of the bradyzoite antigens by the human B-cell response. Serum collected 1 month postinfection had specific antibodies against both the BAG1 and the MAG1 antigen fragments, indicating that the humoral response against bradyzoites and tissue cysts occurs very early after infection. Other studies with an animal model also revealed that tachyzoites differentiate into bradyzoites within a few days after infection (4, 5, 8). This stage of conversion might take place early during human infection. It should also be mentioned that bradyzoite and cyst proteins are released from ingested parasites within the gastrointestinal tract during primary infection. Thus, the host immune response against bradyzoite antigens could originate from this first exposure.

In conclusion, MAG1 is expressed during both tachyzoite and bradyzoite development and is not a bradyzoite-specific marker. This highlights the care that must be taken when proteins secreted in different developmental stages are studied by molecular techniques. The results from our study suggest that MAG1 should rather be considered as a marker specific for acute toxoplasmosis. The final usefulness of the T. gondii MAG1 recombinant antigen as a diagnostic tool should be further investigated by combining different recombinant antigens to increase the sensitivity and the specificity of ELISAs. In our previous studies (13, 23), we indicated that GRA7, GRA6, and P35 recombinant antigens detected IgG antibodies more frequently in serum samples from patients with early T. gondii infection than in serum samples from individuals with chronic T. gondii infection. We now consider including MAG1 in the group of markers that are specific for acute toxoplasmosis. More extensive studies are, however, required and will be performed to strengthen and specify the findings presented in this study.

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