Natural adaptive immune responses in humans against Toxoplasma gondii and Herpes simplex virus type I

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Chapter 7

IgM recognition of recombinant Toxoplasma gondii antigens by sera of acutely or latently infected humans.

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Abstract

Clinical non-relevant (CNR) IgM specific for Toxoplasma gondii is responsible for false-positive results in commercially available IgM assays. Using IgM immunoblotting, it is possible to distinguish between IgM in sera of acutely infected (AI) patients and CNR IgM. Especially the combination of staining of a 55 and 30 kD antigen in T.gondii lysate proved useful in this respect. The 55 kD antigen was identified as Rop1, while the 30 kD antigen was confirmed to be Sag1. However, the use of recombinant antigens instead of lysates for diagnostic assays would improve reproducibility. IgM recognized recombinant Rop1, but most CNR sera also had low anti-Rop1 titers. Although purified native Sag1 separated AI and CNR sera very well on immunoblot, IgM did not recognize recombinant Sag1 at all. Clearly, it is difficult to produce a recombinant Sag1 that can be recognized by IgM. Recombinant Rop1 might be suitable as one of the recombinant antigens in an IgM immunoblot assay, but has to be combined with at least one other immunogenic antigen.
Toxoplasma gondii (Tg) is a ubiquitous protozoan parasite that infects many individuals world-wide. Although the symptoms of infection are usually benign, especially Tg infection during pregnancy can cause life-threatening complications to the fetus. Tg serology is essential to determine whether a pregnant woman is experiencing seroconversion. Detection of anti-Tg IgM is one of the hallmarks of seroconversion [42]. However, one of the problems of Tg serology is the existence of clinically non-relevant (CNR) Tg specific IgM antibodies that persist in sera of individuals who have been infected many years ago [2-4,31]. Our previous experiments demonstrated that many commercially available IgM tests give positive results with well-defined sera containing CNR IgM antibodies [31]. In combination with low IgG titers, sera with CNR IgM are easily confused with those from acutely infected (AI) individuals, and have caused unwarranted concerns and terminations of pregnancy [5]. It has also been demonstrated that CNR IgM antibodies recognize the same antigens as those frequently recognized by IgM in sera of AI individuals [19,31], making it very difficult to devise a toxoplasma antibody test based on specific antigens that will distinguish CNR from AI sera.

Interestingly, using standard immunoblotting (IB) of non-reduced Tg antigens it is possible to distinguish CNR from AI sera based on differences in intensity of recognition of a 30 kD antigen, most likely Sag1. In addition, a combination of intense recognition of the 30 kD antigen and one other antigen, either a GPI anchor or an unidentified 55 kD antigen, proved very useful in the distinction between CNR and AI sera [31]. Sag1 is one of the first antigens recognized by IgM during acute infection, along with the GPI anchor [15,17]. Detection of Sag1 specific IgM is regarded as clinically relevant, and an anti-Sag1 IgM specific ELISA is available [43]. Although our previous results indicated that CNR IgM antibodies recognize Sag1 as well, IgM IB is clearly not as sensitive to anti-Sag1 CNR IgM as commercially available assays. Therefore, IgM IB should be used as a confirmation assay in cases of doubt. However, the use of an incompletely defined Tg lysate as antigen source may cause reproducibility problems in anti-Tg IgM staining patterns due to batch differences, while maintenance of Tg parasites is laborious and expensive. To establish IgM IB as a confirmation assay, it is important to use a standard set of well-defined recombinantly produced antigens.

The objective of this study was to test whether a selected set of immunoblotted recombinant antigens would result in an easy and reproducible confirmation assay for the serology of toxoplasmosis. Identification and cloning of the Tg 55 kD antigen may result in a very useful candidate for an IgM IB assay. In addition, recent expression techniques has allowed the production of purified, recombinant Sag1 recognized by IgG antibodies in human sera [44], indicating that recombinant Sag1 protein has adopted a conformation required for recognition by antibodies. This preparation of recombinant Sag1 together with a recombinant version of the 55 kD antigen and IgM IB may allow the important distinction between sera containing CNR or AI IgM. Before exploring this, it is important to exclude that in some cases other Tg antigens with an approximate molecular weight (MW) of 30 kD are also involved in the anti-Tg IgM response. Moreover, it is not known whether IgM specific for free GPI anchor is also able to recognize GPI-anchored Sag1. Therefore, it is critical to determine if recognition of the 30 kD antigen on immunoblot is solely due to Sag1.

Material & Methods

Sera: All sera used in this study were submitted for routine toxoplasmosis screening. Sera were stored at 20°C. Sera of patients suffering from AIDS or sera of immuno-compromised patients were excluded.

Antigen preparation: Antigen preparation was performed as described elsewhere [8]. The protein concentration of the supernatant (lysat/sonicate) was measured using the Bradford assay with BSA as a standard.
The sonicate was frozen in small aliquots containing 200 μg protein (equal to 7.7×10⁶ tachyzoites) and kept at −70°C until use.

**Primers and cloning of PCR products:** Primers used for amplification of open reading frames encoding Rop1 by PCR and Gra2 by RT-PCR: Rop-FW 5’GAGACCATGCTTCCTGAGCCACAATGGA3’, Rop-RV 5’GAGAGAATTCTTGCGATCCATCATCCT3’, Gra-FW 5’GAGAGGATCCCCATGTTTCGCGTAAACATG3’ and Gra-RV 5’GAGAGGTACCTTACTGCGAAAAGTCTGGGA3’.

PCR products were ligated into an appropriately digested expression vector pRP261, a derivative of vector pGEX-3X (Amersham-Pharmacia).

**Immunoadsorption purification of native SAG1 and production of recombinant SAG1:** Native SAG1 was immunoaffinity purified as described earlier [45]. Recombinant SAG1 produced in *Pichia pastoris* was kindly provided by Alain Jacquet [44].

**Expression and purification of recombinant GST-Rop1, GST-Gra2 and GST:** All proteins were expressed in *E.coli* strain BL21. Before expression induction, BL21 were grown until OD₆₀₀ : 0.8-1 at 37°C. Expression was induced by isopropyl-β-D-thiogalactopyranoside (1 mM) for 2 hours at 37°C. Upon centrifugation at 3500 g for 10 min, cells were resuspended in PBS (pH 7.4) with protease inhibitors (Complete, Boehringer), 1 mM EDTA, 1 mM dithiotreitol and 1% (v/v) Triton X-100. Next, the suspension was freeze/thawed three times and sonicated eight times for 15 sec (Soniprep 150) on ice. Insoluble components were removed by centrifugation (16000g, 10 min, 4°C), and subsequent filtration (0.45 μm). GST fusion proteins were purified and eluted from the supernatant using Glutathione Sepharose 4B beads according to the manufacturer’s instructions (Amersham-Pharmacia). The amount of each recombinant protein used for immunoblots was normalized based on intensity of Coomassie staining on gel and staining with anti-GST antibody (Sigma) on immunoblot (see below).

**SDS-PAGE and Western blotting:** SDS-PAGE was performed as described [9]. 200 μg of the sonicate or 10 μg of purified protein was suspended in SDS-PAGE sample buffer (without reducing agent) to a final volume of 200 μl, boiled for 2 minutes, and loaded onto 10% SDS-PAGE gels. A broad range marker (Bio-Rad Laboratories, Hercules, CA) was included. After electrophoresis, proteins were transferred to polyvinylidifluoride (PVDF) membranes (Millipore) overnight. Transferred proteins and markers were visualised using Ponceau-red dye staining. Blocking buffer consisted of Tris-buffered saline (TBS: 50 mM TrisHCl, 150 mM NaCl, pH 10) containing 0.5% Tween20 and 2% non-fat milk powder.

**Immunostaining of peroxidase conjugated antibodies:** Except when stated otherwise, all samples and conjugates were diluted in TBS containing 0.5% Tween20 and 0.03% non-fat milk powder (TBS-T). All incubations were done using a multiscreeen apparatus (Mini-Protean II, Bio-Rad Laboratories). Isotype specific antibodies conjugated to peroxidase were obtained from DAKO (DAKO, Glostrup, Denmark). The conjugates were diluted 1:5000. All incubations were performed at room-temperature for 75 min. The serum samples of the patients were diluted 50x (unless stated otherwise), incubated, and tested for the presence of anti-*T.gondii* IgM. A mouse monoclonal antibody against SAG1 (HyTest, Turku, Finland) was diluted 1000x, monoclonal Tg49 specific for Rop1 (kindly provided by Dominique Soldati, Heidelberg) was diluted 500x, Gra2 specific monoclonal (gift from Marie-France Cesbron-Delauw, Grenoble) was diluted 10000x. Chemiluminescent substrate was prepared according to the manufacturers descriptions (ECL, Amersham Life Science, Amersham Pharmacia Biotech, Essex, UK). The membranes were incubated with chemiluminescent substrate for 1 min, and exposed to X-ray film for various time-intervals.
**Chapter 7**

A MM

M WW CN R 9

55kD > >

30kD > >

GP II anchor > >

Heaat inactivate d ser a of AI 1 and CNR 5 were dilute dd as indicated , and mixed with a serum that contained IgM antibodies recognizing a 45 kDa T.gondii antigen (dilute d 1/1000). The diluted sera were mixed sequentially (lanes labeled ‘1st’ and ‘2nd’, see method section) with either 2x10^7 parasites in PBS or PBS only. Control lanes represent IgM staining patterns after two incubations with PBS only.

**Preincubation with intact parasites:** Parasites were purified as described above, resuspended in TBS, and counted. Serum samples were heated for 30 minutes at 56°C to inactivate complement, diluted in TBS, mixed with 50 μl PBS containing 2x10^7 purified parasites and incubated for 30 min under gentle rotation. Parasites were pelleted (800xg, 5 min) after incubation and supernatants were split: half was immediately incubated with blots (fraction 1), the rest was incubated once more for 30 min with fresh parasites. After centrifugation, this double absorbed supernatant was incubated with the blots as well (fraction 2). As negative control, samples were treated similarly, but mixed with buffer without parasites (c). Before fractions were incubated with blots, Tween-20 and milk powder were added to final concentrations of 0.5% and 0.03%, respectively. Bound antibodies were detected by chemiluminescence.

Preincubations with purified recombinant Gra2, Rop1 and GST were essentially performed as described for intact parasites; 2 μg recombinant protein was used for each incubation.

**Immunofluorescence (IF):** IF was performed as described [26], except parasites were not fixated. In short, 10^5 filter-purified, freshly egressed parasites were incubated with mouse monoclonal antibodies against SAG1 (HyTest, Turku, Finland) and Rop1 (Tg49), diluted 500x and 250x, respectively, in PBS containing 2% BSA, at 4°C for 30 min. Following 4 washes, parasites were incubated with an anti-mouse Fab conjugated to indocarbocyanine (Cy3) (Jackson Immunoresearch Laboratories, West Grove, PA), diluted 750x, for 30 min. Following washing and drying, labelled parasites were embedded in Vectashield (Vector Laboratories, Burlingame, CA) and visualized using a fluorescence microscope (Leica DMRE/RD, Leica, Wetzlar, Germany) equipped with a camera. Alternatively, parasites were dried on slides and fixated with either aceton (-20°C) or 3% pafurmaldehyde, 0.05% glutaraldehyde at room-temperature as described [46,47,47]. Fixated parasites were exposed to the same monoclonals.

**Elution of IgM antibodies:** Fragments of lysate blots containing either GPI anchor or antigens with MWs ranging between 28-32 kDa were incubated with sera
**Recombinant antigens recognized by IgM**

**Results**

The 55 kDa antigen is Rop1: IgM antibodies are usually directed against surface antigens, therefore preabsorption using intact parasites was performed to determine if the 55 kDa antigen recognized predominantly by AI sera, has surface-exposed epitopes. As shown in figure 1 preabsorption indeed resulted in disappearance of the 55 kDa band, demonstrating that this antigen is present on the surface of tachyzoites. A monoclonal specific for Rop1 also stained an antigen at 55 kDa, so, being a candidate-antigen, Rop1 was cloned and expressed as glutathione-S-transferase (GST)-fusion protein in *E.coli*. In addition to full length GST-Rop1 (~85 kDa), expression and purification resulted in various smaller truncated forms in the range of 70-83 kDa (not shown). Clearly, GST-Rop1 is highly susceptible to proteolytic degradation, as described earlier, possibly due to its unusual charge distribution [46,48]. Sera from acutely and latently infected individuals known to contain anti-55 kDa IgM were incubated successively with 2 μg of the purified recombinant Rop1. In each case, this resulted in gradual decrease and final disappearance of 55 kDa antigen staining from the anti-toxoplasma IgM staining patterns (figure 2), identifying these frequently found IgM antibodies in sera of acutely infected individuals as anti-Rop1 antibodies.

Rop1 is not specifically recognized as a surface-antigen [49]. However, immunofluorescence experiments using the monoclonal specific for Rop1 on intact parasites indeed showed a homogeneous surface staining of approximately 5% of the parasites (figure 3 A-B-C). Once fixated, all parasites demonstrated the expected apical

![Figure 3](image-url)
staining pattern following incubation with the anti-Rop1 monoclonal (figure 3 D-E-F; [49]). Homogeneous staining of fixated parasites was not observed.

_Potentially confounding factors:_ SAG1 and the GPI anchor are known to be major targets of the AI and CNR anti-toxoplasma IgM antibodies in humans. As SAG1 is a GPI-anchored membrane protein we wondered whether IgM antibodies specific for the GPI anchor contribute to the intense signal at 30 kDa. By elution of IgM antibodies recognizing either GPI anchor or SAG1 from blots, and subsequent testing of these eluates on a lysate blot, it was demonstrated that these antibodies only recognized the antigen they were initially eluted from (figure 4).

The 30 kDa signal may not only result from SAG1 recognition, as the Gra2 specific monoclonal stained an antigen at 30 kDa as well, although Gra2 is supposed to run at approximately 28 kDa. The monoclonal proved to be Gra2 specific as recognition was efficiently abrogated following preabsorption with purified, recombinant GST-Gra2 (Figure 5).

**Recognition of a panel of recombinant _T.gondii_ antigens by IgM:** Based on earlier results [31] and the experiments described above, a number of antigens were selected for testing with a panel of extensively characterized sera from AI and CNR individuals on immunoblot. First, it was of interest to determine with this panel of sera if recombinant Rop1 recognition could distinguish between AI and CNR sera. Secondly, we wanted to dissect the IgM signal at 30 kDa that allowed distinction between AI and CNR individuals on _T.gondii_ lysate IB. Thus this panel of sera was tested on blots containing purified, native SAG1, recombinant Rop1, recombinant GST-Gra2 (figure 6), recombinant Sag1 and GST alone (not shown).

Recognition of recombinant Rop1 by AI and CNR IgM coincided with recognition of the 55 kDa antigen on a toxoplasma lysate immunoblot using shorter exposures (≤ 10 min, figure 6, compare upper and middle panel). Longer

![Graph showing IgM Staining Pattern](image)

Figure 4: IgM antibodies recognizing GPI anchor do not cross-react with SAG1.

IgM antibodies in serum of AI 5 strongly recognize GPI anchor and SAG1 (lane A). Serum diluted 200x, incubated with either a toxoplasma lysate blot containing antigens ranging in MW from 28 to 38 kDa (a.o. SAG1, B) or a blot containing antigens of up to 6 kDa (GPI anchor, C) was tested upon elution, using the conditions indicated (see method section) and neutralized. Samples were applied to a whole lysate blot and stained for IgM antibodies (lanes B’; SAG1 and C’, GPI anchor, respectively).

![Graph showing Staining Pattern after Elution](image)

Figure 5: Gra2 and SAG1 comigrate.

The specificity of Gra2 staining was investigated by sequential incubation of both monoclonals with recombinant GST-Gra2. Monoclonal specific staining on a toxoplasma lysate blot following preabsorption is shown: anti-Gra2: lanes 2 and 3, anti-SAG1: lane 3 (only shown after two rounds of preabsorption rounds). Controls are sequential incubations with GST (lanes 4) or with PBS (lanes 1).
Recombinant antigens recognized by IgM

Figure 6: A Comparison of purified antigen and *T. gondii* lysate recognition by Al and CNR IgM.

Diluted sera were applied to blots containing purified recombinant (GST-Rop1 and GST-Gra2) and native (nSAG1) Toxoplasma proteins or total lysates (T.gondii lysate). Besides full-length protein (~83 kDa), expression of Rop1 resulted in various truncated versions of Rop1, ranging mainly between 70 and 83 kDa, hence the ladder of IgM bands in the rRop1 lanes.

Exposures (≥ 60 min) revealed that many samples showed an anti-Rop1 staining IgM pattern that coincided with recognition of the 55 kDa antigen, except for samples CNR 7 and CNR 12 (figure 6, compare second panel above and middle panel).

Most of the Al sera with an intense signal at 30 kDa also intensely recognized native SAG1, while none of the sera displayed strong recognition of recombinant Gra2 (figure 6, compare lower three panels). Three sera showed a weaker anti-SAG1 (Al 4, Al 10 and CNR 7) signal than expected, which may be due to recognition of a third antigen at approximately 30 kDa, while one sample had an overall weak response to the purified proteins (Al 5). In contrast, CNR sera never intensely recognized native SAG1. Except for 1 sample (CNR10, figure 6), recognition of native SAG1 by CNR IgM was as could be expected on the basis of IgM patterns on lysate immunoblot. CNR IgM never bound recombinant Gra2. None of the sera displayed reactivity on immunoblot against GST, or recombinant SAG1 (results not shown).

**Discussion**

In this study we tested whether a selected set of immunoblotted recombinant antigens would result in an easy and reproducible confirmation assay for toxoplasmosis serology. Immunoblotting is one of the few available methods allowing a clear distinction between CNR and Al IgM. The 55 kD antigen preferentially recognized by IgM in Al sera [31], has now been identified as Rop1. This protein belongs to a group of antigens discharged from the rhoptries during Tg invasion and is involved in the formation of the parasitophorous vacuole [50]. Samples from both groups with a clear signal at 55 kD on Tg lysate immunoblot indeed recognized recombinant Rop1. On longer exposure many other serum samples displayed reactivity against rec Rop1, especially in the CNR IgM group, despite the
absence of a signal at 55 kD in the IgM staining patterns analyzed. These anti-Rop1 antibodies might have been missed in our earlier analysis [31], as the exposures used were too short to allow detection of low titers of anti-Rop1 IgM.

Surprisingly, IgM antibodies specific for Rop1 were preabsorbed using intact parasites, while Rop1 is not known to be a surface antigen. Our finding was supported by surface staining of a minority of the intact parasites by the Rop1 monoclonal. Experiments with fixed parasites demonstrated exclusive anti-Rop1 staining of the anterior part of the parasite as described earlier [49,51]. Studies that describe localization and function of Rop1 during infection generally have used fixed cells, which might explain how surface staining was missed previously. The function of Rop1 at the parasites surface remains enigmatic.

Rop1 (P66) is a known target of IgM antibodies during acute infection, and has been selected for an anti-Tg IgM ELISA based on recombinant antigens [52]. Our results indicate that Rop1 as single antigen is not suitable for diagnostic assays, as anti-Rop1 antibodies may cause persistent backgrounds due to intense recognition by CNR IgM. In combination with other recombinant antigens, however, Rop1 may still be valuable in an IgM IB assay as CNR IgM generally recognizes only 1 antigen intensely.

As expected, the signal at 30 kD observed in previous experiments can, in most cases, be attributed to anti-Sag1 IgM. Surprisingly, Gra2 turned out to be a potential confounding antigen, despite its predicted lower molecular weight. The observation that Gra2 and Sag1 comigrate might be a peculiarity of the tricine gels. Although anti-Gra2 antibodies constitute part of the anti-Tg IgG repertoire and recombinant Gra2 is very well recognized by IgG [53], recombinant Gra2 was hardly recognized by IgM and could thus be excluded as potential confounder. This confirms earlier findings that Gra2 is not a major target of anti-Tg IgM antibodies [52].

Another potential contribution to the signal at 30 kD could stem from anti-GPI anchor IgM. In a Tg lysate, Sag1 is known to be GPI anchored [54,55]. Two glycoforms of the Tg GPI anchor have been described of which type B contains the unique and immunogenic glycosylated N-acetylgalactosamine side chain [56]. Using monoclonals, at least two epitopes have been identified on the GPI anchor that are sensitive to periodate and phosphatidylinositol specific phospholipase C treatment [15,57]. These GPI specific monoclonals do not stain membrane antigens on a lysate immunoblot [57], indicating that these epitopes are not available when the GPI anchor is coupled to a membrane antigen. This does not exclude that a polyclonal (human) anti-GPI response could generate cross-reactive antibodies. Nevertheless, our results indicate that also during a polyclonal response anti-GPI antibodies do not stain Sag1.

Following exclusion of confounding factors, we finally tested a recombinant version of Sag1 that is recognized by IgG antibodies in sera of latently infected individuals [44]. This indicates that at least part of the recombinant Sag1 is assembled in the proper conformation for IgG recognition. Unfortunately, IgM did not recognize this recombinant Sag1. Other attempts to incorporate recombinant Sag1 in IgM diagnostic assays have failed probably due to the complex structure of native Sag1 [52]. Importantly, recognition by IgM strongly relies on protein conformation, which is dependent on proper disulfide bridges [45]. The conformation of Sag1 results from a fine-tuned assembly interplay by several Tg chaperones and oxidoreductases. Although there is a report that mentions recognition by AI IgM of cloned Sag1 [58], it is difficult to express properly folded Sag1 in the large amounts required for standardized diagnostic assays.

IgM in two sera appeared to recognize another antigen at 30 kD next to Sag1. A possible candidate that we have not tested is the P35 antigen that runs also at approximately 31 kD under non-reduced conditions [24]. Recombinant P35 performed well in an anti-Tg IgM ELISA [52,59] and could be an alternative to Sag1 to be tested in an IgM IB assay.
Potentially important for the next generation of anti-Tg IgM assays are attempts to produce the GPI anchor *in vitro* [60]. Our previous results argue against the use of the GPI anchor as a single antigen in an assay, but in combination with other cloned antigens it will certainly be of value.

Recombinant Rop1 might be suitable as one of the recombinant antigens in an IgM immunoblot assay, but has to be combined with at least one other, more immunogenic antigen like Sag1. Although immunoblotted native Sag1 can distinguish very well between AI and CNR sera, it clearly is difficult to make recombinant Sag1 recognizable by IgM. Promising alternatives are recombinant P35 and synthetic GPI anchor.