Experimental Parasitology 126 (2010) 263–266

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Research Brief

Evaluation of the antigenic value of recombinant *Toxoplasma gondii* HSP20 to detect specific immunoglobulin G antibodies in *Toxoplasma* infected humans

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ARTICLE INFO

Article history:
Received 31 July 2009
Received in revised form 9 March 2010
Accepted 20 April 2010
Available online 28 April 2010

Keywords:
Toxoplasma gondii
HSP20
Humoral response
Recombinant antigen
Diagnosis

ABSTRACT

Recombinant *Toxoplasma gondii* small heat shock protein HSP20, surface antigen SAG1 and dense granule GRA7 were analyzed by IgG-ELISA with serum samples of *Toxoplasma* infected humans grouped as I (IgG+, IgM+), II (IgG+, IgM−) and III (IgG−, IgM−). rHSP20 reacted against 80% and 62.5% of serum samples from groups I and II, respectively. rSAG1 was recognized by 85% of the samples from group I and 70.8% from group II, whereas rGRA7 was recognized by 85% and 66.6% of the serum samples from groups I and II, respectively. When a combination of two or three recombinant antigens was used, the sensitivity values improved to 85–95% for group I and 87.5–91.7% for group II. All combinations tested produced similar reactivity profiles. None of the recombinant proteins reacted against group III serum samples. In conclusion, we demonstrated that *T. gondii* HSP20 elicits an important B-cell response during human infection, and could be suitable for the development of serodiagnosis tools.

1. Introduction

The protozoan parasite *Toxoplasma gondii* is an important human and veterinary pathogen (Tenter et al., 2000). Because of the late development of the cellular immune response during fetal maturation, *T. gondii* has long been associated with congenital birth defects (Wong and Remington, 1994) and considered a cause of life-threatening opportunistic diseases in immunocompromised individuals as well (Luft and Remington, 1992).

During infection, this parasite elicits a strong humoral and cellular response which protects the host against re-infection (Denkers and Gazzinelli, 1998). Consequently, the detection of specific immunoglobulin G (IgG) antibodies along with absence of the acute-phase markers IgM and IgA allow the diagnosis of the chronic (Ch) stage of infection or a past exposure to the parasite, whereas detection of IgM and IgA suggests an active or recently acquired (RA) infection (Wong and Remington, 1994). However, since IgM antibodies may remain detectable for more than 1 year after initial infection, their detection cannot be used as a marker of acute infection (Wong and Remington, 1994).

Most serological tests for *Toxoplasma* require the preparation of parasite antigens from tachyzoites harvested from mice or cell culture systems. The use of recombinant antigens allows to significantly reduce production costs. In addition, the particular diagnostic value of each antigen can be easily studied and more than one defined antigen can be used to facilitate the standardization of diagnostic systems. In an effort to discern the parasite's antigenic mosaic, several authors analyzed the value of different recombinant antigens (Aubert et al., 2000; Li et al., 2000; Nigro et al., 2003; Pietkiewicz et al., 2004; Beghetto et al., 2006; Holec et al., 2008; Holec-Gasior et al., 2009). These investigations showed that several antigens elicit a humoral response during parasite infection in humans, but with different reactivity in serological assays. Interestingly, some of these antigens are also attractive vaccine candidates (Jongert et al., 2009). Among these antigens, dense granule antigen 7 (GRA7) detected IgG antibodies present in the early phase of infection (Jacobs et al., 1999; Li et al., 2000; Nigro et al., 2003; Pietkiewicz et al., 2004). Another antigen, the surface antigen 1 (SAG1) has been proven to be a good candidate for diagnosis (Harning et al., 1996; Wu et al., 2009), but difficult to express as soluble protein in bacteria (Makioka and Kobayashi, 1991; Chen et al., 2001; Nigro et al., 2003; Meek et al., 2003).

Recently we identified five *T. gondii* small heat shock proteins (de Miguel et al., 2005). One of them, identified as HSP20, is an inner membrane complex associated chaperone (de Miguel et al., 2008). Interestingly, antibodies against *Babesia* HSP20 block parasite growth (Montero et al., 2008). Here, we decided to analyze the reactivity of a recombinant HSP20 protein (rHSP20) with serum samples from pregnant women infected with *Toxoplasma*. Since recombinant proteins SAG1 and GRA7 (rSAG1 and rGRA7) have

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doi:10.1016/j.exppara.2010.04.013

been shown to be valuable antigens for human serodiagnosis (Harning et al., 1996; Aubert et al., 2000; Nigro et al., 2003), we compared the data value of rHSP20 with those obtained with rSAG1 and rGRA7. Finally, we analyzed different combinations of these proteins to determine whether a specific combination can improve the IgG detection in human serum samples from infected individuals.

2. Materials and methods

2.1. Cloning of recombinant proteins

The DNA region encoding the aminoacid residues 77–322 (Harning et al., 1996) of SAG1 (GenBank accession No. AF132217) was amplified by PCR from the recombinant amplicon pZPVXSAG1 (Clemente et al., 2005). The following primers were used: sense 5′ gaattcttactcttaagtgtcctaagacagctcttaca and antisense 5′ gatatctcacatagcaaagatggaaacatgagaagctgt. EcoRI and EcoRV sites (underlined sequences) were included in the sense and antisense primers respectively. The *sag1* DNA fragment was cloned into the prokaryotic expression vector pRSET B (Invitrogen Life Technology), downstream and in frame with a sequence that encodes an N-terminal 6xHis-tag. *Escherichia coli* Rosetta(DE3)pLysS (Novagen) cells were transformed with the expression plasmid.

The cloning procedure and features of rGRA7 and rHSP20 have already been described (Nigro et al., 2003; de Miguel et al., 2005). Briefly, gra7 was cloned into pQE (Qiagen) and expressed in M15 cells (Qiagen), whereas HSP20 was cloned into pRSET (Invitrogen Life Technologies) and expressed into BL21(DE3)pLysS bacteria (Novagen).

2.2. Expression and purification of recombinant proteins

Transformed *E. coli* were grown in LB broth with ampicillin (100 μg/ml) whereas BL21 and Rosetta derived bacteria (rHSP20, rSAG1) were grown in LB broth with ampicillin (100 μg/ml) and chloramphenicol (32 μg/ml). For protein expression, cultures were grown to OD600 = 0.6, and induced by the addition of isopropyl thio-β-D-galactoside (1–2 mM). Bacteria expressing rGRA7 and rHSP20 were lysed with lysis buffer pH8 (300 mM NaCl, 50 mM $NaH_2PO_4{\cdot}H_2O$, 10 mM imidazole). Lysates were sonicated, centrifuged at 10,000 × *g* for 20 min at 4 °C and the supernatants were collected and directly loaded onto a Ni–NTA column previously equilibrated with lysis buffer. The column was washed four times with washing buffer (300 mM NaCl, 50 mM $NaH_2PO_4{\cdot}H_2O$, 30 mM imidazole). Bound recombinant proteins were eluted with elution buffer (300 mM NaCl, 50 mM $NaH_2PO_4{\cdot}H_2O$, 250 mM imidazole). rSAG1 was purified similarly as described above, except that the transformed cells were treated with urea lysing buffer pH 8 (100 mM $NaH_2PO_4{\cdot}H_2O$, 10 mM Tris–HCl, 8 M urea), the column was washed with washing buffer containing urea (100 mM $NaH_2PO_4{\cdot}H_2O$, 10 mM Tris–HCl, 8 M urea, pH 6.3) and rTgSAG1 eluted with 100 mM $NaH_2PO_4{\cdot}H_2O$, 10 mM Tris–HCl, 8 M urea, pH 4.5. The purity of the recombinant proteins was confirmed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis. Finally, protein concentration was determined by the Bradford's method (Bradford, 1976).

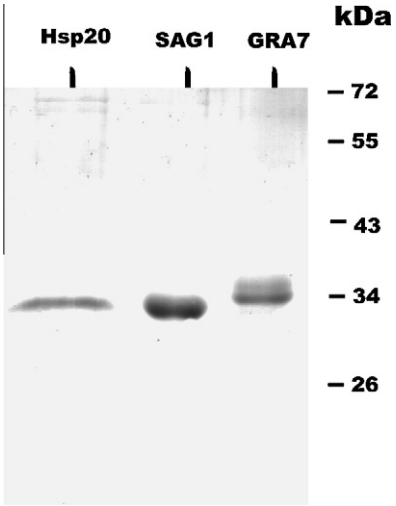

Fig. 1. Expression of *T. gondii* recombinant antigens. Recombinant proteins were expressed fused to a 6-His tag at the N-terminus in *E. coli*, purified by Ni–NTA resin, run onto a SDS–PAGE and stained with Coomassie brilliant blue. On the right side, molecular weight markers are expressed in kDa.

2.3. Serum samples

All sera used in this study were from pregnant women. Serum samples were obtained during routine serologic screening of preg-

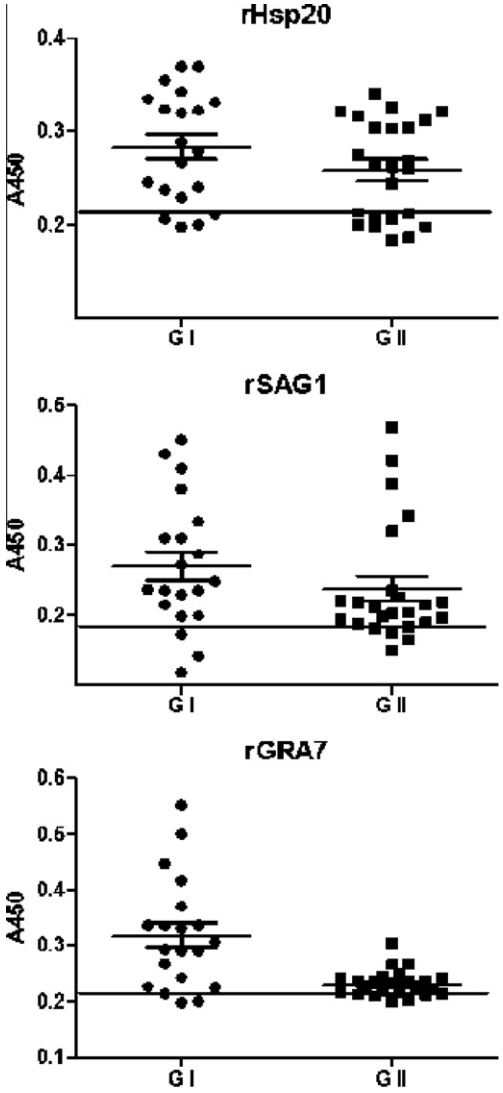

Fig. 2. Immunoreactivity of *T. gondii* recombinant antigens. ELISA microplates were sensitized with rSAG1, rGRA7, rHSP20 and incubated with sera from *Toxoplasma* infected pregnant women from G I and G II. Optical density was measured at 450 nm (A450). Cut-off value (horizontal line) was determined as the mean of the 25 sera from seronegative individuals plus three standard deviations. Positives were considered as such when A450 values were higher than cut-off.

nant women at the ANLIS Dr. Carlos G. Malbran, Argentina, analyzed by IgG-Indirect immunofluorescence assay (IFA) and in-house test IgM-ISAGA. They were grouped as I: IgG+, IgM+ (stage of infection undetermined, $n = 20$); II: IgG+, IgM− (past infection, $n = 24$); and III: IgG−, IgM− (seronegative individuals = 26). Sera from group (G) I have IgG titer higher than 512 besides being IgM+, which could be indicative of individuals with acute infection but also with an early chronic infection (mainly within the first year). Serum IgG-titers samples obtained from G II ranged between 32 and 1048. In addition, 25 seronegative samples were used to determine the cut-off values. Samples were stored at −20 °C until use.

2.4. IgG-Enzyme-linked immunosorbent assay (ELISA)

Polystyrene 96-well microtitre plates (Nalge Nunc International) were coated with 2–5 μg/ml of different recombinant proteins diluted in 50 mM carbonate buffer (pH 9.6) and incubated overnight at 4 °C. Plates were blocked for 1 h at 37 °C with blocking solution (5% milk in 100 mM Tris–HCl, pH 7.5, 0.9% NaCl, 0.1% Tween 20 [TBS–Tween]) and washed three times with TBS–Tween prior to incubation with serum samples. One hundred microliters of serum dilution in blocking solution was added to each well per duplicate. Plates were placed in a humidified chamber and incubated for 1 h at 37 °C and then washed three times. One hundred microliters of a 1/5000 dilution of peroxidase-conjugated goat anti-human IgG (Sigma–Aldrich) diluted in blocking solution was added to each well, and incubated for 30 min at 37 °C. After three washes, the peroxidase activity was detected with H_2O_2 and *o*-phenylenediamine dihydrochloride (OPD, Sigma–Aldrich, Argentina) for 30 min at room temperature. The reaction was stopped by the addition of 1 N H_2SO_4. Optical density (OD) at 450 nm was read in a microplate reader (Kayto model RT-2100C).

3. Results and discussion

T. gondii SAG1, GRA7 and HSP20 fused to 6His-tag were expressed as recombinant (r) proteins in *E. coli* and purified (Fig. 1) as previously described (Nigro et al., 2003; de Miguel et al., 2005; Harning et al., 1996). rSAG1 was optimally purified as a soluble recombinant protein, yielding 50 mg/l culture, under denaturing conditions.

In order to test the reactivity of human serum samples against HSP20, rHSP20 was analyzed with human serum samples by ELISA. The analysis showed that rHSP20 reacted against 80% (16/20) and 62.5% (15/24) of serum samples from G I (G I) and G II (G II), respectively (Fig. 2). No serum samples from G III reacted against HSP20. rSAG1 and rGRA7 were used as highly reactive *Toxoplasma* antigens (Harning et al., 1996; Jacobs et al., 1999; Aubert et al., 2000; Nigro et al., 2003). rSAG1 showed reactivity against 85% (17/20) and 70.8% (17/24) serum samples from G I and G II, respectively (Fig. 2). rGRA7 showed reactivity against 85% (17/20) and 66.6% (16/24) of serum samples from G I and G II, respectively (Fig. 2). In conclusion, rHSP20 presented a slightly lower reactivity level than these recombinant antigens.

Based on the fact that the three antigens presented a high antigenic value, a combined ELISA was performed. The recombinant antigens were mixed in combination of three or two and assayed against human serum samples. As observed in Fig. 3, the four combinations produced a similar reactivity profile and sensitivity val-

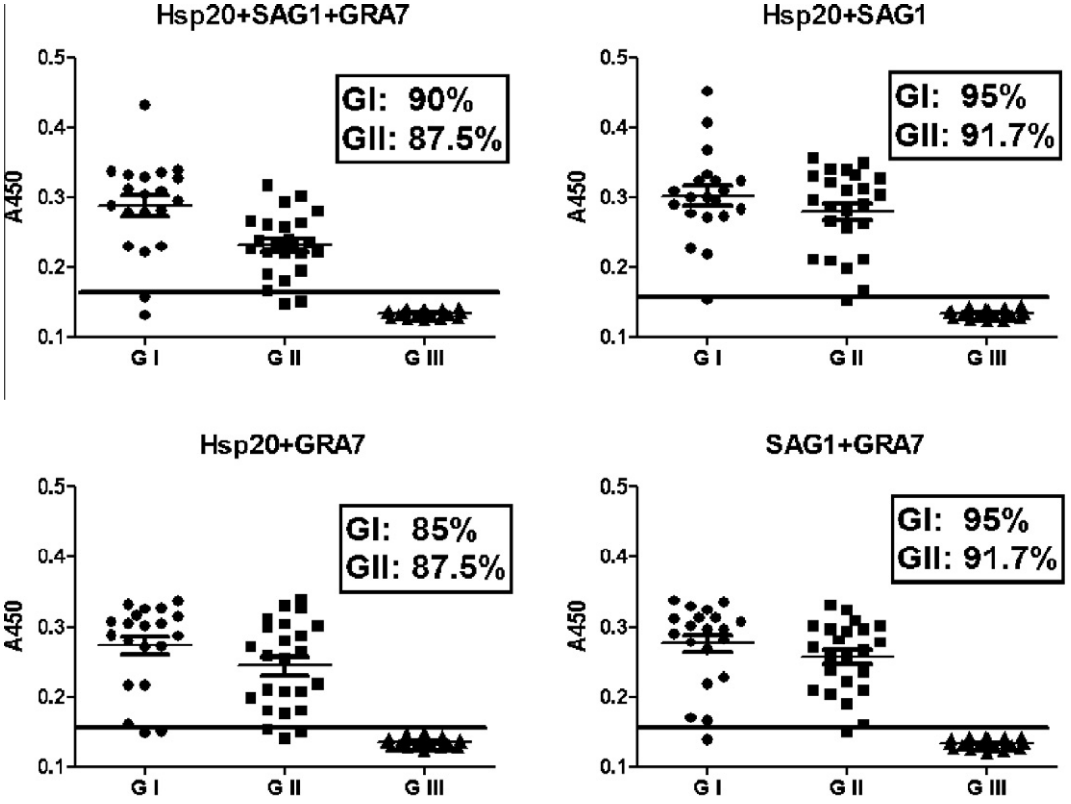

Fig. 3. Immunoreactivity analysis of IgG-mixELISAs. ELISA microplates were sensitized with 2 μg of rSAG1, rGRA7 and rHSP20 (HSP20 + SAG1 + GRA7) or 3 μg of rHSP20 and rSAG1 (HSP20 + SAG1) and incubated with sera from *Toxoplasma* infected pregnant women from G I and G II and from seronegative individuals (G III). Optical density was measured at 450 nm (A450). Cut-off value (horizontal line) was determined as the mean of the 25 sera from seronegative individuals plus three standard deviations. Positives were considered as such when A450 values were higher than cut-off.

ues ranging between 85–95% for G I and 87.5–91.7% for G II. The mix of two or three antigens improved clearly the sensitivity of G II in comparison to single antigens as SAG1 or GRA7 (Figs. 2 and 3). These results could be due to the fact that the IgG titers from G I sera are higher than 512, while titers from G II range from 32 to 1024. In the first case, the amount of antibodies present in the serum samples seems to be enough to give a high reactivity to a sole recombinant antigen, whereas in the second case the combination of SAG1 with one or more antigens, seemed to be necessary to increase antibody detection. The combination of the three antigens did not improve the ELISA performance in comparison with a mix of two antigens. By contrast, the mix of the three antigens gave a slightly low reactivity value than some combinations of two antigens. A possible explanation of this result is that the addition of the three antigens produced some interference which would impair the correct coating of some of them. None of the G III serum samples produced any reactivity against the different mixes (Fig. 3).

Holec et al. (2008) showed that the reactivity against Mic1ex2 was detected in 100% of serum samples from recently infected individuals, but sera from chronic infections presented 75% reactivity. The combination of different antigens (mix 1: GRA1 + -GRA7 + SAG1, mix 2: P35 + SAG2 + GRA6 and mix 3: rMic1ex2 + MAG1 + MIC3) allowed to increase the sensitivity value of sera from the chronic group to 88.9%, 94.4% and 88.9%, respectively. These data, together with the results here presented show that while sera from individuals with high IgG and IgM titers with either acute or chronic infection, anti-*Toxoplasma* antibodies can be detected with high sensitivity with a sole recombinant antigen, the combination of different antigens with high antigenic value is crucial for the detection in sera from individuals with past infection and negative IgM.

The main finding of this study is that a high number of pregnant women presented antibodies against HSP20 at least at the beginning of the infection, indicating that this chaperone elicited an important B-cell response during human toxoplasmosis. Previously, antibodies against *Babesia* HSP20 were shown to block parasite growth (Montero et al., 2008). Coincidently, preliminary results from our group also showed that anti-*T. gondii* HSP20 antibodies could partially block parasite invasion (unpublished results). *T. gondii* rHSP20 could be a promising vaccine target candidate, complementing the T-cell response conferred by other antigens. A series of experiments are currently being carried out to shed light on this issue. Another important finding of this work is that HSP20 presented a similar antigenic behavior than other recognized *Toxoplasma* antigens such as rGRA7 and rSAG1, and that its use in combination with other *Toxoplasma* recombinant antigen can improve serological detection of toxoplasmosis. Despite the fact that additional work with a larger number of human serum samples is required to confirm these data, we can conclude that HSP20 is a novel *T. gondii* antigen of diagnostic value.

Acknowledgments

S.O. Angel (Researcher), M.M. Corvi (Researcher), M. Clemente (Researcher), V. Coceres (Fellow) and M. Laguia Becher (Fellow) are members of National Research Council of Argentina (CONICET). This work was supported by: ANPCyT grant BID OC-AR PICT 05-11266 (to S.O.A.), BID OC-AR PICT 05-34415 (to S.O.A.) and BID OC-AR PICT 04-25286 (to M.Cl.). We thank Mara Rosenzvit for critical reading of the manuscript and Bibiana Ledesma for the serum samples.

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