Reactivity of Synthetic SAG1 (p30) Peptide Sequences with RH, S273 and Beverley Strain-Induced Anti-*Toxoplasma gondii* Antibodies

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Key Words

IgG antibodies · SAG1 · Synthetic peptides · *Toxoplasma gondii*

Abstract

Objectives: We compared the reactivity of IgG1 and IgG2a antibodies in mouse sera after infection with virulent RH and low-virulent S273 and Beverley strains of *Toxoplasma gondii* against RH SAG1 recombinant p30 (rp30) and synthetic SAG1 peptides. **Methods:** Infected mouse serum samples were collected 9 days after infection, and the level of total IgG, IgG1 and IgG2a against the RH SAG1 rp30 protein and twenty peptides of the RH SAG1 protein were assessed. The glycosylphosphatidylinositol (GPI) modification site, the hydrophilic-hydrophobic structure, the transmembrane region and the secondary structure of the SAG1 sequence of virulent and low-virulent strains were analyzed using software. **Results:** The virulent strain-infected mice produced a higher level of IgG1 but a lower IgG2a against the rp30 antigen, while the low-virulent strain-infected mice produced a higher level of IgG2a than the virulent strain. The difference in the secondary structure of SAG1 protein between the virulent and low-virulent strain was largely confined to amino acid positions 291–336, showing mutations and GPI anchor site. **Conclusion:** The difference in the reactivity of IgG against the rp30 antigen and synthetic peptides between virulent and low-virulent strains points to the importance of the pri-

mary and secondary structure assumed by antigens in the activation of Th cells and, subsequently, in the induction of IgG and its subclasses.

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Introduction

Toxoplasma gondii is an obligate intracellular Apicomplexa parasite ubiquitous in a broad range of warm-blooded animals, including humans. Although it causes asymptomatic or relatively mild infection in healthy adults, it can result in abortion or neonatal malformations as well as severe disease and death among immunodeficient and immunocompromised individuals [1, 2]. Several excretory-secretory and stage-specific surface antigens of bradyzoites, tachyzoites and sporozoites have been reported to play an important role in the mechanism(s) of pathogenesis and immunity against *T. gondii* infection [3, 4]. *Toxoplasma* infection was indicated by higher IFN- γ and TNF- α in the serum level of C57BL mice than in that of BALB/c mice [5]. Th1 cells produce IL-2 and IFN- γ and control the production of IgG2a, whereas Th2 cells produce IL-4, IL-5, IL-6 and IL-10 and also control the production of IgG1 and IgE [6]. SAG1, a well-known major surface antigen, together with a family of other related parasite surface coat proteins, is involved in the attachment and/or regulation of the host

Table 1. SAG1 peptide sequences designed from the RH SAG1 antigen (accession No. CAA32244)

Peptide, No.	Sequence	aa region
1	SDPPLVANQVVTC PDKKS	48–65
2	DKKS TAAVILTPTEN NHFT	62–79
3	NHFT LKCPKTALTE PPTLA	76–94
4	PPTLA YSPNRQIC PAGTTS	90–108
5	AGTTS SCTSKAVTLSS LIP	104–122
6	LIPEA EDSWWT GDSASL	120–136
7	GDSASL DTAGIKLTV PIEKF	131–150
8	PIEKF PVTTQTFFW GCIKG	146–164
9	GCIKG DDAQSCMVT TVQA	160–178
10	TVQA RASSVVNNV ARCSYG	175–193
11	RCSYG ADSTLGPVK LSAEG	189–207
12	LSAEG PTMTLV OGKGVK	203–221
13	KDGVK VPQDN QYCSGTTL	217–235
14	SGTTL TGCNEK SFKDILPK	231–249
15	DIPKL TENPWQ GNASSDK	245–263
16	ASSDK GATLT IKKEAFP AE	273–291
17	AFP AE SKSVI IGCT GGSPE	273–291
18	GGSPE KHHCTVK LEFAGAA	287–305
19	FAGAA GSAKSAAGT ASHVS	301–319
20	ASHVS IFAMVIGLIG SIAACVA	315–336

Each peptide has a 3- to 6-aa overlap (in bold) before and after the sequence.

immune response [7]. SAG1 attaches to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor and constitutes 3.0–5.0% ($1.5\text{--}2.5 \times 10^6$ copies) of the total number of surface membrane proteins expressed per cell [8]. The GPI-anchored SAGs are immunogenic and share common primary sequence features [9–11]. In this study, we compared the reactivity of IgG subclasses in sera of mice infected with the virulent and low-virulent strains of *T. gondii* against recombinant p30 (rp30) and synthetic SAG1 peptides to obtain an indirect estimation of the induction of Th1 and Th2 cell responses.

Materials and Methods

T. gondii Infection

Low-virulent S273 and Beverley strain cysts were obtained from brain tissue of chronically infected mice. An in vitro cultured virulent RH strain was used. Per parasite strain, 3 female ICR mice, 6–8 weeks old (Nihon Clea, Tokyo, Japan), were intraperitoneally inoculated with approximately 20 cysts of the Beverley and S273 strains and 1×10^4 tachyzoites of the RH strain. Serum samples were collected on day 9 after infection and assayed.

Peptide Synthesis

Twenty peptides of the RH SAG1 protein (accession No. CAA32244) were designed comprising a 3- to 6-amino acid (aa) overlap before and after the sequence (table 1), but without the N-terminal signal sequence (aa 1–46) domains. Peptides were synthesized using the Fmoc method at Sigma Genosys (Ishikari, Japan). The reactivity of the peptides with anti-*T. gondii* IgG1 and IgG2a was compared using serum samples of virulent- and low-virulent-infected mice.

Construction of RH Strain Recombinant SAG1 (rp30) in *Escherichia coli*

The recombinant protein of *T. gondii* SAG1 (accession No. M23658) was prepared as described by Kato et al. [12]. Briefly, the SAG1 ORF region of the *T. gondii* RH strain was ligated with the pGEX-4T vector (Amersham Pharmacia, Uppsala, Sweden), and the expressed recombinant proteins were purified according to the manufacturer's instructions. Thrombin protease (Amersham Pharmacia) was used to cleave the glutathione S-transferase fusion protein.

Indirect ELISA

An indirect ELISA was performed according to a previously described procedure [12]. Microtiter wells (Maxisorp, Nunc, Roskilde, Denmark) coated with 100 μ l of 2 μ g/ml rp30 or 2 μ M of synthesized peptide were used. Sera from *T. gondii*-infected mice serially diluted in PBS (rp30 reactivity, 1/100–1/10,000; synthesized peptide reactivity, 1/500) were added, incubated at 37°C for 1 h and washed 3 times in T-PBS. Per well, 100 μ l of horseradish peroxidase-conjugated anti-mouse IgG (H+L), IgG1 or IgG2a were added and incubated at 37°C for 1 h, and the reactivity was visualized with a TMB-stabilized substrate (Dako Japan, Kyoto, Japan) and stopped with 1 N H_2SO_4 . The optical density was read at 450 nm. Data are presented as means $\pm$ SE.

Secondary Structure Prediction of SAG1 (RH, S273 and Beverley Strains)

The GPI modification site and the transmembrane region of the SAG1 sequence of virulent and low-virulent strains [13] were analyzed using free software available online. The GPI modification site (http://www.mendel.imp.univie.ac.at/gpi/cgi-bin/gpi_pred.cgi) and the transmembrane region (<http://sosui.proteome.bio.tuat.ac.jp>) were predicted using both types of software. The secondary and hydrophilic-hydrophobic structure of the SAG1 protein (aa sequence 1–336) was searched and analyzed using Genetyx Software (Genetyx, Tokyo, Japan) [14, 15].

Results

The titers of anti-*T. gondii* IgG and its subclasses, IgG1 and IgG2a, in serum samples of mice infected with the virulent and low-virulent strains against the rp30 antigen are shown in figure 1. The level of IgG production was slightly lower in the sera of the virulent strain-infected mice (fig. 1a). The level of IgG2a production was comparable in the virulent and low-virulent strain-in-

Fig. 1. ELISA for anti-*T. gondii* IgG Abs in serum samples of RH, S273 and Beverley strain-infected mice against RH rp30. Three serum samples were collected and assayed. Each point represents the mean of three samples $\pm$ SE. **a** Total IgG Ab titer. **b** IgG1 Ab titer. **c** IgG2a Ab titer.

fectured mice (fig. 1c). Virulent strains had a lower level of IgG2a production than low-virulent strains. They show an acute and a lethal phase after infection; as a result, the level of IgG2a, which is driven from Th1, is lower than that in low-virulent strains. The titers of IgG1 and IgG2a in serum samples of virulent and low-virulent *T. gondii*-infected mice were compared for their reactivity with each of the 20 RH SAG1 synthetic peptides. The low-virulent strain-infected mice sera showed relatively higher reactivity of IgG1 against peptides 1–4 and 8 (aa 48–108 and 146–164, respectively), whereas the virulent strain showed much higher reactivity against peptides 8 and 20 (aa 315–336, fig. 2a). The IgG2a production level of the low-virulent Beverley strain was much higher in peptides 5 (aa 104–122), 8, 19 (aa positions 301–319) and 20 than in other peptides. On the other hand, the low-virulent S273 strain had relatively higher reactivity of IgG2a against peptides 1, 8 and 17–20 (aa 273–336, fig. 2b). Interestingly, IgG1 of the virulent strain and IgG2a of the low-virulent strain (fig. 2) demonstrated much higher reactivity to peptides 8 and 20.

Genetyx software analysis was used to probe into the structural relationship of SAG1 peptides inducing Th2-driven IgG1 or Th1-driven IgG2a synthesis; the SAG1 protein of both virulent and low-virulent strains revealed differences largely confined to sequence fragment aa 291–336 (fig. 3). In peptide 17, the difference was noted in the aa 291 terminus, which translated to an α -helix secondary structure in the low-virulent strain and in a coiled-coil secondary structure in the RH strain. Peptide 18 in the low-virulent strain exclusively had the α -helix structure, while peptide 20 shared a similar secondary structure. Interestingly, in both the virulent and low-virulent forms, peptide 19 (aa 301–319) consisted of a coiled-coil, α -helix and β -sheet structure, but there was a preponderance of the coiled-coil structure in the low-virulent strain (fig. 3). In addition, on the basis of a loose-parameter-condition analysis, the probability of the β -turn-commencing region is higher in peptide 19 of the low-virulent strain (fig. 3). In addition, on the basis of the GPI modification results, the SAG1 GPI anchors are located near aa 306, and the best GPI cleavage site is predicted at aa 310 (fig. 4). Similarly, the SAG1 protein has three mutations

Fig. 2. Comparison of the reactivity of 20 synthesized RH SAG1 peptides with IgG Abs in sera of mice infected with different strains of *T. gondii*. Three serum samples were collected per group and assayed. Each point represents the mean of three samples $\pm$ SE. **a** IgG1 Ab titer. **b** IgG2a Ab titer.

Fig. 3. Secondary structure predicted from aa 291–336 in RH and S273 (S) and Beverly strains (B) of *T. gondii*. AA = Amino acid sequence; 2nd = predicted secondary structure; a = α -helix; b = β -sheet; c = coiled coil; + = probability of β -turn commencing region. Amino acid mutations are underlined.

	291		336
AA	EKHHCTV <u>KL</u>	EFAGAAGSAK	<u>S</u> AGTASHVS IFAMVIGLIG SIAACVA
RH			
2nd	ccbbbbb	aaaaaacaaa	aaaacccbbb bbbbbbba aaaaaa
		++++	
AA	EKHHCTV <u>QL</u>	EFAGAAGSAK	<u>S</u> AGTASHVS IFAMV <u>T</u> GLIG SIAACVA
B, S			
2nd	aaaaaaaa	aaaaaacccc	ccccccbbb bbbbbbba aaaaaa
		+++++	+++++

Fig. 4. Prediction of transmembrane region of SAG1 (accession No. 32244). Predicted transmembrane sequences are underlined. N-terminal signal sequences are bold. SAG1 GPI anchors are located near aa 306 (arrow), and the best GPI cleavage site is predicted at aa 310 (bold arrow).

	10	20	30	40	50	60
	MSVSLHHFII	SSGFLTSMFP	KAVRRAVTAG	VFAAPTLMSP	LRCGVMASDP	PLVANQVVT
	70	80	90	100	110	120
	PDKKSTAAVI	LTPTENHFTL	KCPKTALTEP	PTLAYSPNRQ	ICPAGTTSSC	TSKAVTLSSL
	130	140	150	160	170	180
	IPEAEDSWWT	GDSASLDTAG	IKLTVPIEKF	PVTTQTFFVG	CIKGDDAQSC	MVTVTVQARA
	190	200	210	220	230	240
	SSVNNVARC	SYGADSTLGP	VKLSAEGPTT	MTLVCGKDG	KVPQDNNQYC	SGTTLTGNE
	250	260	270	280	290	300
	KSFKDILPKL	TENPWQNGAS	SDKGATLTIK	KEAFPAESKS	VIIGCTGGSP	EKHHCTVKLE
	310	320	330	340		
	FAGAAGSAKS	AAG TASHVSI	FAMVIGLIGS	IAACVA		
	↑	↑				

in sequence aa 291–336, which translated to a difference in the secondary structure of peptides 18 and 19 between the virulent and the low-virulent strains (fig. 3).

Discussion

Toxoplasma is a strong inducer of antigen-specific CD4+ and CD8+ T lymphocytes, indicating that parasite peptides are efficiently targeted at the appropriate cellular pathways of antigen presentation during infection. In vivo, SAG1 stimulates natural killer (NK) cell proliferation and activity, induces IFN- γ and IL-12 expression [16–18] and elicits protection that is largely cytotoxic T cell dependent [19–23]. We have reported significant in vitro activation and synthesis of cytokines IFN- γ , IL-10 and IL-12 by T cells from immunized mice in the presence of rp30 antigen [12]. Similarly, earlier in vivo studies have shown Th2-driven IgG1 and Th1-driven IgG2a antibody (Ab) synthesis in *T. gondii*-infected mice [23, 24], the production of IgG2a, which had been linked to the activation of IFN- γ -producing Th1 cells, and the cooperation between Th2 and B cells in the generation of IgM, IgG1, IgA and IgE [25]. In this study, the sera of RH strain-infected mice against the rp30 protein revealed a lower titer of all IgGs. The low-virulent strain showed higher reactivity of IgG1 against the hydrophilic region from peptides 1–4 and 8, since the reactivity of these peptides was higher than the average range of peptides 1–20 in low-virulent strains, whereas the virulent strain showed higher reactivity against hydrophilic peptide 8 and hydrophobic peptide 20. Peptides 8 and 20 appear to be important antigens for inducing Th2-driven IgG1 responses in an acute-phase virulent-strain infection. For low-virulent strains, hydrophilic peptide antigens 1–4 are a Th2-driven IgG1-inducible region. On the other hand, the higher IgG2a production level of low-virulent strains mainly focuses on the hydrophobic and transmembrane regions from peptides 17–20. Peptides 8 and 20 demonstrated a much higher reactivity with IgG1 of the virulent strain and IgG2a of the low-virulent strain.

The virulent strains are more lethal than the low-virulent strains in the early stage of infection, which is attributed to the difference in the Th cell-inducible antigen region, which results in differences in the induction of immunity between virulent and low-virulent strains during infection. We estimated that the differences in the Th cell-inducible antigen in the virulent and low-virulent strains are related to the secondary structure and GPI anchor. It was highly discernable in peptide 19, with that

in the low-virulent strain comprising more of the disordered coiled-coil structure caused by the Ala³¹¹→Ser³¹¹ polymorphism [26].

The polymorphism in peptide 18 (aa 298), however, resulted in a change in the secondary structure to the α -helix in the low-virulent strain against the β -sheet in the virulent strain. The presence of the C-terminus coiled-coil structure in peptide 17 and a more disparate secondary structure in peptide 18 of the RH strain contrasted with the exclusively α -helix structure of the low-virulent form. Maki et al. [13] reported mutations in the virulent strain SAG1 aa 263, 270 and 298 Lys positions were changed to Asn, Asn and Gln, respectively, in the low-virulent strain and effected a reduction in molecular surface-positive charge. In addition to the higher probability of a β -turn commencing region in peptide 19 of the low-virulent strain, its hydrophobicity is predictably higher relative to that of the RH strain. Fiorentino et al. [6] reported that T cell antigenic sites are stabilized by hydrophobic interactions that can enhance antigen presentation on the cell surface and subsequent recognition by T cells.

The surface of *T. gondii* tachyzoites and bradyzoites is covered with GPI-anchored proteins [27], many of which are members of the SAG families [10, 12], playing different roles in host cell invasion, immune modulation and/or virulence attenuation, as well as parasite survival [15]. Earlier studies on SAG1 transgenic mice have demonstrated protection similar to that induced in the wild type following infection with a lethal dose of the low-virulent Beverley strain [22].

Subtle differences in the structure of SAG1 GPI anchor proteins are believed to exist among RH and other strains [18]. There is a Ser³¹¹→Ala³¹¹ polymorphism, which also predicted the GPI anchor site in peptide 19 between the low-virulent P(LK) strain [28] and the virulent RH strain. The algorithm of Kodukula et al. [29] was applied to the deduced amino acid sequence of SAG1 from RH. Furthermore, Zinecker et al. [30] also suggested that the GPI anchor of SAG1 from the RH strain has at least two possible glycoforms. In a mature SAG1 sequence, the latter part of the 19 and 20 peptide sequence encoded a transmembrane domain. Until GPI modification, peptides 19 and 20 are retained on the membrane [4], and predicted GPI cleavage sites of the RH strain in peptide 19 are amino acid point mutations in low-virulent strains. After GPI anchoring, amino acid lengths retained on the membrane are different and unregulated in the RH strain. This structural feature may have influenced epitope recognition, and binding with Abs in each strain resulting

in IgG2a reactivity of each infected strain showed relatively higher reactivity to peptides 19 and 20. Hansen et al. [31] have reported that Ab formation of GPI-anchored proteins in response to malaria infection can arise by both major histocompatibility complex (MHC) II dependent and independent pathways and relate with CD1d-restricted NK T cells. CD1-restricted NK cells enhance parasite-specific Abs and affect immune responses toward Th2 cells. In murine malaria infections, Th1 responses that switch to Th2 are noted 7–10 days after infection, being regulated by CD1-restricted NK cells. Ab formation of *Plasmodium* non-GPI- and GPI-anchored antigens is associated with MHC class II molecules [32]. Antigen presentation mechanisms of MHC class I during natural infection is little known, but SAG1 antigen presentation by MHC class I can elicit CD8 responses [33]. Splenocytes from SAG1-immunized mice had increased levels of CD8+ T cells, which were found to kill extracellular parasites [34]. While SAG1 can act as a target of CD8+ T cells during infection, other *T. gondii* antigens are likewise able to elicit cytotoxic T lymphocytes (CTL) activity. CD8+ effectors require the presence of CD4+ T lymphocytes, although CD4+ T lymphocytes are not generally considered major cytotoxic effectors. For anti-parasite effectors, CD4+ T lymphocytes produce IL-2 that generates CD8+ lymphocyte effector functions, e.g. CTL activity and IFN- γ production. Nielsen et al. [35] predicted four CTL epitopes, aa 276–283 (peptide 17), aa 155–162 (peptide 8), aa 239–246 (peptide 14) and aa 324–331 (peptide 20), respectively, and peptide-MHC interaction was performed. Each peptide showed MHC binding, but only aa 276–283 (peptide 17) provided protection against *T. gondii* by CD8+ T lymphocytes.

Moreover, the higher antigenicity of SAG1 peptides with IgG1 compared to that with IgG2a is indicative of a significantly heightened Th2 cell induction and, presumably, an enhancement of cytokine synthesis, which is essential in cell-mediated protective immunity against *T.*

gondii. It has been reported that Th1 cells produce large quantities of IFN- γ and mediate classical cellular immunity. However, Th2 cells are involved in humoral immunity and produce IL-4, IL-5 and IL-13. IL-23 derived from naive CD4+ T cells and IL-17 derived from Th17 played different roles in host defense against infection [36]. IL-17-producing effector T cells are important in inflammatory responses and independent of the mechanisms required for Th1 and Th2 cell development [37]. In human and normal mouse T cells and monocytes, IFN- γ synthesis is inhibited by IL-10, while IL-12 induces Th1 cell activation [38]. During the initial phase of *T. gondii* infection, IL-10 and IL-12 have been reported as major antagonists in the regulation of IFN- γ synthesis [39]. The present findings corroborate earlier data on the in vivo studies on SAG1 inducing IFN- γ and IL-12 synthesis providing largely cytotoxic T cell-mediated protection. The induction of high levels of IFN- γ and both IL-10 and IL-12 expression particularly by SAG1 is clearly suggestive of the activation of protective immunity. IL-10 and IL-12 are primarily involved in host defense, and IL-23 and IL-17 are important in the defense against extracellular pathogens.

In summary, the difference in the reactivity of IgG against the rp30 antigen and synthetic peptides between virulent and low-virulent strains points to the importance of the primary and secondary structures assumed by antigens in the activation of Th cells and, subsequently, in the induction of IgG and its subclasses. In view of the present findings, it would be interesting to conduct immunization experiments with *T. gondii* strains using SAG1 synthetic peptides.

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