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Research brief

Toxoplasma gondii: P30 peptides recognition pattern in human toxoplasmosis

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ABSTRACT

In this study, human sera reactivity against nine peptides derived from the *Toxoplasma gondii* P30 protein was assessed by ELISA in patients with different clinical forms of toxoplasmosis. Same as has been reported in mice, sera from congenital, ocular and chronic asymptomatic toxoplasmosis patients recognized more strongly peptides from the protein's carboxy-terminus, being peptide 2017 (amino acids 301–320) the one most strongly recognized by sera from patients with ocular toxoplasmosis. Serum samples collected from 13 patients without ocular infection, 13 with inactive chorioretinal scars, 6 with active ocular infection and 10 seronegative individuals were then screened for anti-2017 IgG. Peptide 2017 was recognized by all patients' samples but not by sera from *T. gondii*-seronegative individuals. No statistically significant differences were found between the absorbance levels of groups with and without lesions or with active or inactive ocular lesions, as determined by ANOVA.

1. Introduction

Infection by *Toxoplasma gondii* is among the most widely distributed protozoan infections in the world. This parasite can infect virtually all mammal and bird species. Infection is usually asymptomatic, but a primary infection during pregnancy can result in neurological and ocular complications in the fetus (Gómez Marín et al., 1997). In addition, ocular complications can occur in a significant percentage of adult persons with recently acquired toxoplasmosis. Thus, in Colombia it has been estimated that more than 5% of the population develop retinocoroideal scars after a non-congenital infection and that 20% of them have reduced visual capacity (de-la-Torre et al., 2007). As the acquired immunity is in most of cases protective, it is important to characterize the underlying mechanisms. Most of antibodies raised during human *Toxoplasma* infection are targeted to the major surface antigen P30 (Velge-Roussel et al., 1994). It has been recently shown that only 8 out of the 17 peptides comprising this protein induce production of specific antibodies in mice, and that only four peptides derived from the carboxyl terminus protect against lethal challenge, conferring significant survival (Siachoque et al., 2006). Such study highlighted the importance of determining the precise sequences against which effective immune responses are directed to. To date, it is still not clear which P30 peptides are recognized by antibodies from humans infected with *T. gondii* (Godard et al., 1994; Velge-Roussel et al., 1994; Beghetto et al., 2003; Graille et al., 2005). In these previous works, all lineal peptides that can be derived from P30 were not examined systematically. Therefore, several immunoassays using selected peptides from the *T. gondii* P30 major protein were carried out in this study, using serum samples from symptomatic and asymptomatic individuals, in order to define a more precise humoral epitope mapping of the P30 protein in humans.

2. Materials and methods

The sequence of *T. gondii* P30 (Genbank Accession No. CAA32245) was divided into 17 non-overlapping 20-mer long peptides. Only the nine antibody inducing peptides reported by previous studies in mice (Siachoque et al., 2006), were chemically synthesized using the solid phase multiple peptide technique (Merrifield, 1963). Peptide sequences are shown in Table 1. MBHA resin (0.49 mEq/g), *t*-Boc and low–high HF cleavage were used in the process (Tam, 1988). Once synthesized, peptides were extracted with 10% acetic acid and water and purified by HPLC. Volatilization was performed in USP grade saline solution, at a final 4 mg/mL concentration and pH was adjusted to 6.8–7.2. Then, dialysis was performed to remove low weight particles using a 12–14 kDa membrane, followed by peptide lyophilization to guarantee stability. Peptides were stored at 4 °C until use. Specific enzyme

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Table 1
Amino acid sequence of the nine *Toxoplasma gondii* P30 protein-derived peptides.

Peptide 2002	(1–20 aa)	M S V S L H H F I I S S G F L T S M F P
Peptide 2003	(21–40 aa)	K A V R R A V T A G V F A A P T L M S F
Peptide 2005	(61–80 aa)	P D K K S T A A V I L T P T E N H F T L
Peptide 2008	(121–140 aa)	I P E A E D S W W T G D S A S L D T A G
Peptide 2009	(141–160 aa)	I K L T V P I E K F P V T T Q T F V V G
Peptide 2011	(181–200 aa)	S S V V N N V A R C S Y G A D S T L G P
Peptide 2014	(241–260 aa)	K S F K D I L P K L T E N P W Q G N A S
Peptide 2015	(261–280 aa)	S D K G A T L T I K K E A F P A E S K S
Peptide 2017	(301–320 aa)	F A G A A G S A K S A A G T A S H V S I

immunoassays for each peptide were performed by coating Maxisorp micro titer plates with 100 μL of each peptide diluted in carbonate buffer pH 9.6 (Na_2CO_3: 0.159 g/100 mL, $NaHCO_3$: 0.293 g/100 mL), incubating plates first for 1 h at 37 °C, then 48 h at 4 °C and finally 1 h at 37 °C. Unspecific ligand sites were then saturated with 100 μL of 2% casein phosphate buffer for 1 h at 37 °C. Plates were then washed once with 0.01 M PBS (pH 7.2) and then incubated with 100 μL of serum, diluted 1:100 in 5% casein phosphate buffer, for 1 h at 40 °C. After washing five times with phosphate buffer plus 0.05% Tween 20, 100 μL of horseradish peroxidase-conjugated anti-mice diluted 1:6000 in 6% casein phosphate buffer were added for 20 min at 37 °C. After five washes with phosphate buffer plus 0.005% Tween 20, the horseradish peroxidase activity was detected by using TMB for 30 min at 37 °C and then stopped by adding a 5% H_2SO_4 solution. Absorbance of duplicates from each serum were read at 450 nm. Positive cut-off point was determined by estimating the mean average absorbance of ten negative controls plus two standard deviations. Serum samples from patients with congenital toxoplasmosis, ocular toxoplasmosis and controls were selected from the serum bank of our laboratory, according to previously described diagnosis criteria (de-la-Torre et al., 2007; Gallego-Marín et al. 2006). A recombinant P30 protein, kindly provided by Dr. Michael Grigg from the Molecular Parasitology section at the NIH, Bethesda, USA, was used to perform a competitive assay in order to evaluate the antibodies' specificity. The recombinant protein was added in increasing concentrations (0, 0.01, 1 and 10 μg) in the presence of the serum, during the incubation phase of the ELISA assay. Statistical tests were performed with the SPSS version 14.0 software (Lead Technologies Inc, USA).

3. Results and discussion

The results of the specific test for antibodies in three children with congenital toxoplasmosis, three patients with ocular toxoplasmosis, one control negative for IgG antibodies and one control positive for IgG antibodies, but asymptomatic and without chorioretinal scars, are shown in Fig. 1. Only five (2005, 2011, 2014, 2015 and 2017) out of the nine peptides tested were recognized by sera from this group of patients, while the absorbances of the remaining peptides were below of the assay's cut-off point. Contrary to what has been reported in mice, human serum antibodies recognized peptide 2005 (amino acids 61–80) that is near to the amino terminus, while the recognition pattern of the remaining peptides was similar, being most of the antibodies directed towards P30 carboxy-terminus. Since the mean absorbance ± standard deviation (ma ± SD) was higher for peptide 2017 (0.28 ± 0.12), compared to the other peptides and these differences were statistically significant -except when compared to peptide 2015- (peptide 2005: 0.15 ± 0.05, $p = 0.003$; peptide 2011: 0.13 ± 0.03, $p = 0.01$; peptide 2014: 0.15 ± 0.02, $p = 0.03$; peptide 2015: 0.22 ± 0.03, $p = 0.15$), we then tested the recognition of this single peptide by a larger group of sera. Furthermore, this peptide had induced the highest antibody levels and conferred the highest protection levels against lethal challenge in a previous study in mice (Siachoque et al., 2006). In total, we assessed 32 serum samples from three groups of anti-*Toxoplasma* IgG positive people having either chorioretinal scars ($n = 13$), an inactive chorioretinal scar ($n = 13$), or chorioretinal active inflammatory lesions at the indirect fundoscopy ($n = 6$). This peptide was recognized by all serum samples at the specific ELISA assay (Fig. 2) and no significant differences were observed between mean absorbances of the three groups, as determined by an ANOVA statistical test. The mean absorbance of the ten anti-*Toxoplasma* IgG negative serum samples was 0.09 ± 0.01, while it was 0.36 ± 0.04 ($p = 0.000$) for the 32 anti-*Toxoplasma* IgG positive samples. No statistically significant differences were found between the mean of absorbances of people without ocular lesions (0.35 ± 0.03) versus those with ocular lesions (0.36 ± 0.04, $p = 0.55$) nor between the mean absorbances of patients with ocular inactive lesions (0.37 ± 0.04) versus those with active ocular lesions (0.34 ± 0.04, $p = 0.85$).

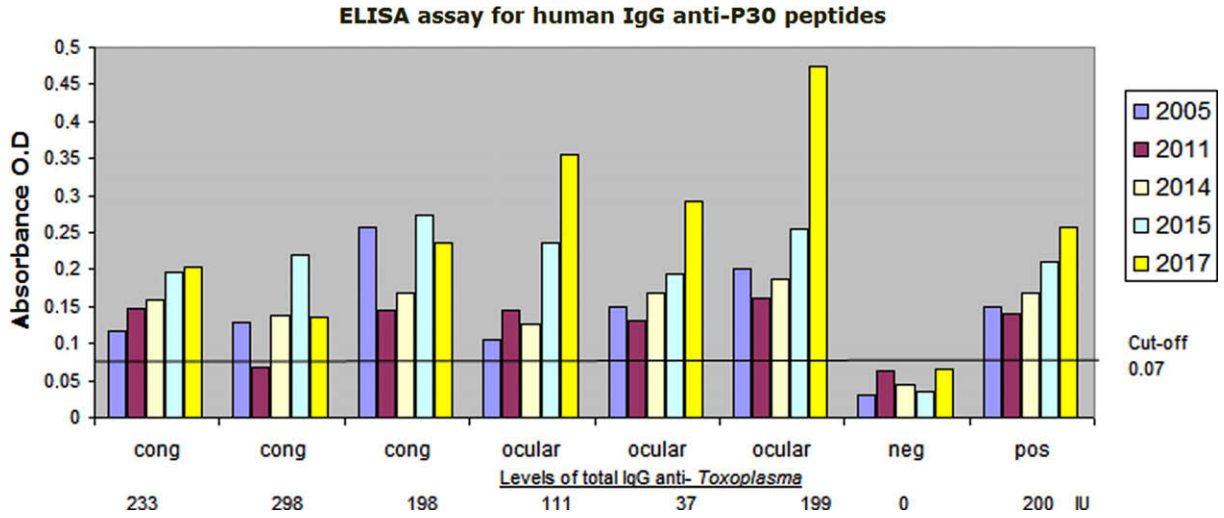

Fig. 1. Absorbances of five P30 peptides assessed using sera from children under 1 year of age with congenital toxoplasmosis (cong), ocular toxoplasmic retinochoroiditis (ocular), one anti-*Toxoplasma* IgG negative person (neg) and one anti-*Toxoplasma* IgG positive person with asymptomatic toxoplasmosis and negative fundoscopy for chorioretinal scars (pos). The results in international units for total IgG specific anti-*Toxoplasma* antibodies, as determined by a commercial ELISA assay (Human, Germany), are shown below each serum.

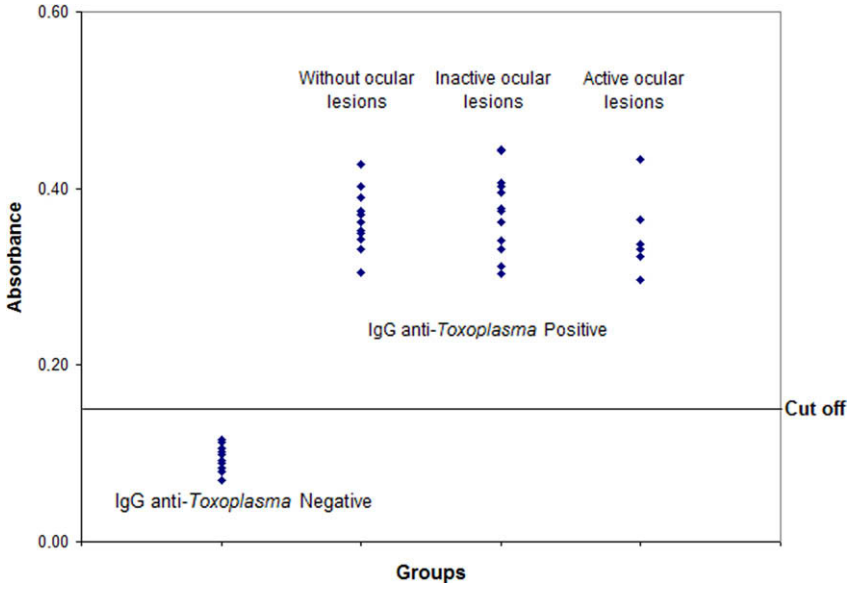

Fig. 2. Absorbances in a specific ELISA of IgG antibodies against the P30-derived peptide 2017 (amino acids 301–320) in four groups of people. The first group comprised sera from ten people previously determined to be anti-*Toxoplasma* IgG negative, and three groups of people with positive anti-*Toxoplasma* IgG tests (without ocular lesions, with active ocular lesions and with inactive chorioretinal lesions, respectively). The cut-off point for the assay is indicated by the horizontal line.

In order to demonstrate that this assay was specific, antibodies were tested in a competition assay between the purified recombinant P30 protein and peptide 2017. The recombinant P30 protein abrogated, in a dose dependent fashion, the reactivity of one serum that was positive against peptide 2017 at the ELISA (Fig. 3).

Our results suggest that there are significant differences among humoral immune responses induced against peptides derived from the major surface protein of *T. gondii* in humans. Previous studies tested a fewer number of P30-derived peptides (Velge-Roussel et al., 1994; Godard et al., 1994) and another one analyzed the immune response to recombinant proteins obtained from phage-display (Beghetto et al., 2003). Nevertheless, these studies led to contradictory results, since while the screening of the *T. gondii* cDNA phage-display library showed that patients' sera recognize more strongly the SAG1 D1 domain (Begheto et al., 2003), the use of SAG1-derived peptides identified the C-terminus of the D2 domain as the major antigenic and immunogenic fragment (Godard et al., 1994). This can be explained due to the fact that the two regions included in the phage clones were too large regions (135 and 152 amino acids, respectively), and only five peptides were assayed in the mouse model. Therefore, these previous studies lack of precision to determine the P30 epitope mapping and are difficult to compare. Our previous studies have systematically examined all the possible regions of this protein

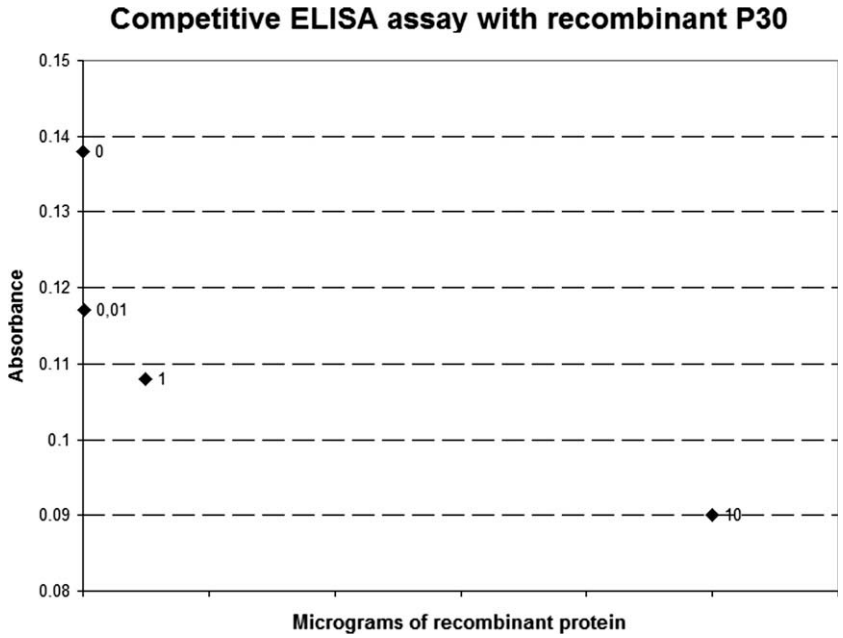

Fig. 3. Competitive assay carried out with IgG antibodies against the P30-derived peptide 2017 (amino acids 301–320) from one positive serum in presence of increasing concentrations of the recombinant P30 protein. The concentration in micrograms of the recombinant purified protein is indicated at each side of the mark that indicates the absorbance. The Spearman test for correlation between absorbance and protein concentration indicated a statistically significant inverse correlation ($p = 0.019$).

and precisely defined which interesting candidate peptides are immunogenic and have a protective inducing ability against lethal challenge in mice (Siachoque et al., 2006). It is important to highlight what the vast experience obtained in the search for a peptide vaccine against malaria has taught us in the precise selection of an immunogenic sequence within a protein (Patarroyo and Patarroyo, 2008). We have focused particularly on peptide 2017 (amino acids 301–320) given that this peptide induced the highest antibodies and protection levels in the mouse model (Siachoque et al., 2006). These results were confirmed by a study carried out by a Japanese group, which applied the Genetyx software to analyze the structural relationship of SAG1 peptides (Kato et al., 2007). Our peptide 2017 (amino acids 301–320) corresponds to peptide 19 (amino acids 301–319) in the nomenclature of Kato et al. They found that differences between the SAG1 protein of virulent and low-virulent strains were mostly confined to the fragment comprising amino acids 291–336. The secondary structure of peptide 19 expressed by virulent and low-virulent strains consisted of a coiled-coil, α-helix and β-sheet structures, although there was higher content of coiled-coil structures in the protein of low-virulent strains. In addition, a loose-parameter condition analysis indicated a higher probability of β-turn-starting regions for peptide 19 of the low-virulent strain. In addition, the most probable GPI cleavage site was predicted to be at amino acid 310. Similarly, three nucleotide substitutions were found in the SAG1 portion encoding amino acids 291–336, which translated into a difference in the secondary structure of peptide 19 between the virulent and the low-virulent strains. Altogether, the data gathered from studies in mice by different authors (Siachoque et al., 2006; Kato et al., 2007) and by the present study using serum samples from humans, indicate the existence of a dichotomy in the immune response induced against *Toxoplasma* P30 protein that can be dissected through the analysis of peptides derived from an immunogenic protein. Our results show that the peptide spanning amino acids 301–320 of P30 was recognized by all the patients with asymptomatic or symptomatic *Toxoplasma* infection. This confirms that detection of antibodies does not predict protectivity against intracellular pathogens. One question that remains unsolved: are there any differences at the cellular immune response level between symptomatic and asymptomatic people, even though all infected people are able to produce antibodies against this peptide?

In conclusion, this work shows that, in humans, anti-P30 antibodies recognizing linear epitopes are directed mostly against peptides from the P30 carboxy-terminus. Additionally, the humoral response against the peptide including amino acids 301–320 is similar in symptomatic and asymptomatic people with *Toxoplasma* infection. Further studies assessing the differences at the cellular immune response level should be performed.

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