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BRIEF COMMUNICATION

Toxoplasma-SPECIFIC IgG SUBCLASS ANTIBODY RESPONSE IN CEREBROSPINAL FLUID SAMPLES FROM PATIENTS WITH CEREBRAL TOXOPLASMOSIS

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SUMMARY

Cerebral toxoplasmosis can be highly debilitating and occasionally fatal in persons with immune system deficiencies. In this study, we evaluated the *Toxoplasma gondii*-specific IgG subclass antibody response in 19 cerebrospinal fluid (CSF) samples from patients with cerebral toxoplasmosis who had a positive IgG anti-*T. gondii* ELISA standardized with a cyst antigen preparation. There were no significant differences between the rates of positivity and the antibody concentrations (arithmetic means of the ELISA absorbances, MEA) for IgG_1 and IgG_2, but the rates of positivity and MEA values for these two IgG subclasses were significantly higher than those for IgG_3 and IgG_4. The marked IgG_2 response in CSF from patients with cerebral toxoplasmosis merits further investigation.

KEYWORDS: Cerebral toxoplasmosis; Cerebrospinal fluid; IgG subclasses.

INTRODUCTION

Human infection with *Toxoplasma gondii* is usually asymptomatic or is associated with mild, non-specific clinical symptoms in the majority of immunocompetent persons. However, toxoplasmosis can be highly debilitating and occasionally fatal in persons with immune system deficiencies and in congenitally infected infants[13,25,30].

Host resistance to *T. gondii* is predominantly controlled by cell-mediated immunity[9], although the humoral immune response may also play an important role[7,11]. In humans, the major antibody class produced in the humoral response to *T. gondii* is IgG[24]. Studies based on immunoenzymatic techniques (ELISA, immunoblot) standardized with tachyzoite antigen preparations have shown that IgG_1 is the dominant IgG isotype involved in humoral response to *T. gondii* in humans[2,7,10,12,17,18].

Determination of the IgG subclass antibody response to *T. gondii* could contribute to our understanding of the pathogenesis of toxoplasmosis, as well as its diagnosis[18]. The cyst stage represents a lifetime risk for the reactivation of *Toxoplasma* infection in immunocompromised individuals[13,16]. The aim of this study was to evaluate the *Toxoplasma*-specific IgG subclass antibody response in cerebrospinal fluid (CSF) samples from patients with cerebral toxoplasmosis who had a positive IgG anti-*T. gondii* ELISA standardized with a cyst antigen preparation.

MATERIAL AND METHODS

Patients and samples: *Toxoplasma*-specific IgG subclasses were evaluated in CSF samples from 19 patients with cerebral toxoplasmosis who were positive for anti-*T. gondii* IgG in CSF by ELISA, using the cyst antigen preparation described below. All patients had clinical and neuroimaging findings compatible with cerebral toxoplasmosis and the brain lesions and symptoms improved after anti-parasitic treatment. The CSF samples of ten patients had a positive nested polymerase chain reaction using primers for the B1 gene. Twenty-five CSF samples from patients with other neurological disorders [multiple sclerosis (n = 10), neurocysticercosis (n = 5), neurosyphilis (n = 3), neurocryptococcosis (n = 3) and bacterial meningitis (n = 4)] were used as controls. The CSF samples from patients with cerebral toxoplasmosis and other neurological disorders were collected for diagnostic purposes. After completion of all the solicited routine tests, the remaining volume of the CSF samples were used to detect *Toxoplasma*-specific IgG subclasses. All patients were attended to the university hospital of the State University of Campinas (UNICAMP), Campinas, Sao Paulo, Brazil. This study was approved by the Ethics Committee of the Faculty of Medical Sciences, UNICAMP, in accordance with the resolutions of the Brazilian National Ethics Committee.

Antigen preparation: cysts from the P strain of *T. gondii*[19] were purified using the procedure described by KASPER (1989) from brains

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of chronically infected female Swiss mice. After purification, the cysts were washed three times by centrifugation in 0.15 M phosphate-buffered saline, pH 7.2 (PBS) and the final pellet was resuspended in this same solution and sonicated for 3 min (1 min sonication/1 min pause) in an ice water bath using a Branson sonicator (model SX-30) at a power setting of 2 with a 20% pulse duty cycle. After sonication, enzyme inhibitors (phenylmethylsulfonyl fluoride - PMSF and leupeptin) were added to the sonicated material to final concentrations of 2.5 µM and 5 mM, respectively. After stirring for two h in an ice water bath, the material was centrifuged (10,000 x *g*, 4 °C, 30 min) and the supernatant was separated and stored in aliquots at -20 °C. This protocol was approved by an institutional Committee for Ethics in Animal Use (CEUA/UNICAMP, protocol no. 3025-1).

ELISAs: serial dilution experiments were performed to determine optimal concentrations of reagents (antigen, monoclonal antibodies and conjugate) to be used in the ELISAs. The wells of polystyrene microtiter plates (Greiner Bio-One, Kremsmünster, Austria) were coated with antigen preparation (1 µg protein/mL in 0.1 M carbonate/bicarbonate buffer, pH 9.5) by incubating for one h at room temperature (RT) and 16 h at 4 ºC. After incubation, the wells were washed three times with PBS containing 0.1% Tween 20 (PBS-T) and, after that, 100 µL of PBS-T containing 0.1% bovine serum albumin were added to the wells. After 30 min incubation at RT, the wells were washed twice with PBS-T and 100 µL of each CSF sample diluted 1:5 with PBS-T were added in duplicate to the wells for one h at RT. Subsequently, the wells were washed three times with PBS-T and 100 µL of monoclonal anti-human IgG_1, anti-human IgG_2, anti-human IgG_3 or anti-human IgG_4 (Sigma, St. Louis, MO, USA) diluted 1:750 in PBS-T were added to each well and the plates were incubated for one h at RT. After washing the wells three times with PBS-T, 100 µL of conjugate (peroxidase-labeled sheep anti-mouse IgG; Sigma) diluted 1:1000 in PBS-T were added to the wells and the plates were incubated for one h at RT. After incubation and three washes with PBS-T, 100 µL of substrate (0.42 mM tetramethylbenzidine and 1.42 mM H_2O_2 in 0.1 M sodium acetate/acetic acid buffer, pH 5.5) were added to the wells. Ten minutes after substrate addition, the reactions were stopped by adding 50 µL of 2 N H_2SO_4 to each well and the resulting absorbances were read at 450 nm in a Multiskan ELISA reader (Labsystems, Helsinki, Finland). Positive and negative controls were included in each plate. Each CSF sample was tested in duplicate and the mean absorbance determined. The final absorbance for each CSF sample was determined by subtracting the mean absorbance of two antigen controls in the corresponding plate. The cut-off value for each assay was determined by a receiver operating characteristic (ROC) curve[15], using the ELISA results obtained with CSF samples from patients with cerebral toxoplasmosis and other neurological disorders.

Data analysis: the rates of positivity and arithmetic means of the ELISA absorbances (MEA) for each subclass were compared using the Cochran Q test[4,14] and repeated measures ANOVA with transformation by ranks and profile test[5,29], respectively, with $p < 0.05$ indicating significance. The statistical analyses were done using SAS (Statistical Analysis System) for Windows version 9.2 (SAS Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Table 1 shows the rates of positivity of the ELISAs for the detection of IgG subclasses, as well as the MEA values for CSF samples from patients with cerebral toxoplasmosis. There were no significant differences between the rates of positivity and the MEA values for IgG_1 and IgG_2, but the rates of positivity and MEA values for these two IgG subclasses were significantly higher than those for IgG_3 and IgG_4 ($p = 0.0042$ and $p < 0.0001$, respectively). The IgG_3 and IgG_4 ELISAs showed the same rate of positivity, but the MEA of the IgG_3-ELISA was significantly higher than that of the IgG_4 ELISA ($p < 0.0001$).

Table 1
Toxoplasma IgG subclass antibody responses in cerebrospinal fluid (CSF) samples from patients with cerebral toxoplasmosis

IgG subclass	Rate of positivity (%) $p = 0.0042$*	MEA $p < 0.0001$**
IgG_1	84.2 (a)	0.163 (c)
IgG_2	73.7 (a)	0.264 (c)
IgG_3	36.8 (b)	0.074 (d)
IgG_4	36.8 (b)	0.041 (e)

MEA = mean of the ELISA absorbances; *Cochran's Q test; **Repeated measures ANOVA and profile test. Values followed by the same letter are not significantly different.

Infection with *T. gondii* is controlled primarily by cell-mediated immunity[9]. Some studies have also shown that the humoral immune response may protect against the parasite[7,11]. Several studies using immunological techniques standardized with tachyzoite antigen preparations have shown that IgG_1 is the dominant IgG subclass involved in the humoral response to *T. gondii* in humans[2,7,10,12,17,18].

The rupture of *Toxoplasma* cysts in the brain may cause disease reactivation and severe encephalitis in immunocompromised hosts[13,16]. However, there is only limited data on the utility of detecting *Toxoplasma*-specific antibodies in CSF samples from patients with cerebral toxoplasmosis. POTASMAN *et al*. (1988) showed that production of anti-*T. gondii* IgG antibodies in the central nervous system may be diagnostic of toxoplasmic encephalitis. CHANDRAMUKHI (2004), using a commercial ELISA kit (Omega Diagnostics, UK), detected *Toxoplasma*-specific IgG antibodies in 92% of CSF samples from autopsies of proven cases of cerebral toxoplasmosis, indicating that the detection of specific antibodies in CSF can be a useful adjunct to clinical and neuroimaging findings for the diagnosis of this neuroinfection. MEIRA *et al*. (2011) showed that the detection of *Toxoplasma*-specific IgG antibodies in CSF samples by immunoenzymatic techniques standardized with *T. gondii* excreted/secreted antigens (ESA), in association with clinical, serological and radiological information, can be useful for diagnosing cerebral toxoplasmosis, particularly in patients with active disease. As shown here, there were no significant differences between the frequency and concentration of *Toxoplasma*-specific IgG_1 and IgG_2 antibodies in CSF samples from patients with cerebral toxoplasmosis based on an ELISA standardized with an antigen preparation from *T. gondii* cysts.

Protein antigens elicit mainly IgG_1 and IgG_3 antibodies, while in adults polysaccharide antigens preferentially induce antibodies of IgG_2[1,27]. The *Toxoplasma* tachyzoite-bradyzoite differentiation is associated with morphological and molecular changes, including the expression of stage-

specific proteins such as surface antigens or enzymes and alterations in parasite metabolism that are probably needed for parasite adaptation to environmental changes[6,21]. Two major changes in the sugar content occur during cyst formation, namely, the synthesis of large amounts of polysaccharide that is stored as amylopectin granules in the bradyzoite cytoplasm and the presence of lectin-binding sugars in the cyst wall[6,28]. The presence of polysaccharides (carbohydrates) in the cyst antigen preparation used in our ELISA could account for the high frequency of IgG_2 detected here.

Some studies have suggested that *Toxoplasm*a-specific IgG subclasses may be markers of congenital and cerebral toxoplasmosis or clinical outcome[2,7,8,23]. In a study using an ELISA standardized with tachyzoite antigens and serum samples from mother/newborn pairs with maternal exposure to *T. gondii*, CAÑEDO-SOLARES *et al*. (2008) showed that IgG_1 in the mothers and IgG_3 in the newborns were related to offspring clinical problems; IgG_2 and IgG_3 in babies were markers of vertical transmission, whereas IgG_4 in mothers or children was associated with clinical problems. DE SOUZA-E-SILVA *et al*. (2013), in a study designated to evaluate the association between clinical signs of congenital toxoplasmosis and IgG subclasses, showed that the detection of anti-rMIC3 IgG_2 and IgG_4 was associated with the presence of retinochoroidal lesions and intracranial calcifications. MEIRA *et al*. (2013) showed that the detection of IgG_4 specific for *T. gondii* ESA in serum and/or CSF supported the diagnosis of cerebral toxoplasmosis in HIV-infected patients.

Our results confirmed the predominance of IgG_1 antibodies in the immune response to *T. gondii*. However, IgG_2 antibodies were found at a frequency and concentration similar to IgG_1 antibodies in CSF samples from patients with cerebral toxoplasmosis. The marked IgG_2 response in CSF from patients with cerebral toxoplasmosis reported here merits further investigation.

RESUMO

Resposta de anticorpos específicos das subclasses da IgG para *Toxoplasma* em amostras de líquido cefalorraquidiano de pacientes com toxoplasmose cerebral

A toxoplasmose cerebral pode ser altamente debilitante e ocasionalmente fatal em pessoas com deficiências do sistema imune. Nesse estudo, nós avaliamos a resposta de anticorpos das subclasses da IgG para o *Toxoplasma gondii* em 19 amostras de líquido cefalorraquidiano (LCR) de pacientes com toxoplasmose cerebral que apresentavam uma reação IgG anti-*T. gondii* positiva com ELISA padronizada com uma preparação antigênica de cistos. Não foram encontradas diferenças significativas entre as taxas de positividade e as concentrações de anticorpos (média aritmética das absorbâncias das reações ELISA, MEA) para IgG1 e IgG2, mas as taxas de positividade e valores MEA para estas duas subclasses de IgG foram significativamente superiores aos da IgG3 e IgG4. A resposta marcante de anticorpos IgG2 em LCR de pacientes com toxoplasmose cerebral merece investigação adicional.

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REFERENCES

1. Barrett DJ, Ayoub EM. IgG_2 subclass restriction of antibody to pneumococcal polysaccharides. Clin Exp Immunol.1986;63:127-34.

2. Cañedo-Solares I, Galván-Ramírez M de L, Luna-Pastén H, Rodríguez Pérez LR, Ortiz-Alegría LB, Rico-Torres CP, *et al*. Congenital toxoplasmosis: specific IgG subclasses in mother/newborn pairs. Pediatr Infect Dis J. 2008;27:469-74.

3. Chandramukhi A. Diagnosis of neurotoxoplasmosis by antibody detection in cerebrospinal (CSF) fluid using Latex Agglutination Test and ELISA. J Commun Dis. 2004;36:153-8.

4. Conover WJ. Practical nonparametric statistics. New York: John Wiley & Sons Inc; 1971.

5. Conover WJ, Iman RL. Rank transformations as a bridge between parametric and nonparametric statistics. Am Stat. 1981;35:124-9.

6. Coppin A, Dzierszinski F, Legrand S, Mortuaire M, Ferguson D, Tomavo S. Developmentally regulated biosynthesis of carbohydrate and storage polysaccharide during differentiation and tissue cyst formation in *Toxoplasma gondii*. Biochimie. 2003;85:353-61.

7. Correa D, Cañedo-Solares I, Ortiz-Alegria LB, Caballero-Ortega H, Rico-Torres CP. Congenital and acquired toxoplasmosis: diversity and role of antibodies in different compartments of the host. Parasite Immunol. 2007;29:651-60.

8. de Souza-e-Silva CH, Vasconcelos-Santos DV, de Andrade GQ, Carellos EV, de Castro Romanelli RM, de Resende LM, *et al*. Association between IgG subclasses against *Toxoplasma gondii* and clinical signs in newborns with congenital toxoplasmosis. Pediatr Infect Dis J. 2013;32:13-6.

9. Denkers EY, Gazzinelli RT. Regulation and function of T-cell-mediated immunity during *Toxoplasma gondii* infection. Clin Microbiol Rev. 1998;11:569-88.

10. Derouin F, Sulcebe G, Ballet JJ. Sequential determination of IgG subclasses and IgA specific antibodies in primary and reactivating toxoplasmosis. Biomed Pharmacother. 1987;41:429-33.

11. Dupont CD, Christian DA, Hunter CA. Immune response and immunopathology during toxoplasmosis. Semin Immunopathol. 2012;34:793-813.

12. Ee TY, Singh M, Yap EH. The determination of anti-*Toxoplasma gondii* antibodies in different IgG subclasses of human sera by the enzyme-linked immunosorbent assay (ELISA). Southeast Asian J Trop Med Public Health. 1989;20:71-9.

13. Feustel SM, Meissner M, Liesenfeld O. *Toxoplasma gondii* and the blood-brain barrier. Virulence. 2012;3:182-92.

14. Fleiss JL. Statistical methods for rates and proportions. 2nd ed. New York: John Wiley & Sons; 1981.

15. Fletcher RW, Fletcher SW. Clinical epidemiology: the essentials. 4th ed. Philadelphia: Lippincott Williams & Wilkins; 2005.

16. Frenkel JK, Escajadillo A. Cyst rupture as a pathogenic mechanism of toxoplasmic encephalitis. Am J Trop Med Hyg. 1987;36:517-22.

17. Giraldo M, Cannizzaro H, Ferguson MA, Almeida IC, Gazzinelli RT. Fractionation of membrane components from tachyzoite forms of *Toxoplasma gondii*: differential recognition by immunoglobulin M (IgM) and IgG present in sera from patients with acute or chronic toxoplasmosis. J Clin Microbiol. 2000;38:1453-60.

18. Huskinson J, Stepick-Biek PN, Araujo FG, Thulliez P, Suzuki Y, Remington JS. *Toxoplasma* antigens recognized by immunoglobulin G subclasses during acute and chronic infection. J Clin Microbiol. 1989;27:2031-8.

19. Jamra LM, Vieira M de P. Isolamento do *Toxoplasma gondii* de exsudato peritoneal e órgãos de camundongos com infecção experimental. Rev Inst Med Trop Sao Paulo. 1991;33:435-41.

20. Kasper LH. Identification of stage-specific antigens of *Toxoplasma gondii*. Infect Immun. 1989;57:668-72.

21. Lyons RE, McLeod R, Roberts CW. *Toxoplasma gondii* tachyzoite-bradyzoite interconversion. Trends Parasitol. 2002;18:198-201.

22. Meira CS, Vidal JE, Costa-Silva TA, Frazatti-Gallina N, Pereira-Chioccola VL. Immunodiagnosis in cerebrospinal fluid of cerebral toxoplasmosis and HIV-infected patients using *Toxoplasma gondii* excreted/secreted antigens. Diagn Microbiol Infect Dis. 2011;71:279-85.

23. Meira CS, Vidal JE, Costa-Silva TA, Motoie G, Gava R, Hiramoto RM, *et al*. IgG_4 specific to *Toxoplasma gondii* excretory/secretory antigens in serum and/or cerebrospinal fluid support the cerebral toxoplasmosis diagnosis in HIV-infected patients. J Immunol Methods. 2013;395:21-8.

24. Montoya JG. Laboratory diagnosis of *Toxoplasma gondii* infection and toxoplasmosis. J Infect Dis. 2002;185(Suppl 1):S73-82.

25. Montoya JG, Liesenfeld O. Toxoplasmosis. Lancet. 2004;363(9425):1965-76.

26. Potasman I, Resnick L, Luft BJ, Remington JS. Intrathecal production of antibodies against *Toxoplasma gondii* in patients with toxoplasmic encephalitis and the acquired immunodeficiency syndrome (AIDS). Ann Intern Med. 1988;108:49-51.

27. Schroeder HW Jr, Cavacini L. Structure and function of immunoglobulins. J Allergy Clin Immunol. 2010;125(2 Suppl 2):S41-52.

28. Sullivan WJ Jr, Jeffers V. Mechanisms of *Toxoplasma gondii* persistence and latency. FEMS Microbiol Rev. 2012;36:717-33.

29. Tabachnick BG, Fidell LS. Using multivariate statistics. 4th ed. Needham Heights: Allyn & Bacon; 2001.

30. Tenter AM, Heckeroth AR, Weiss LM. *Toxoplasma gondii*: from animals to humans. Int J Parasitol. 2000;30:1217-58.

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