

Impact of stage differentiation on diagnosis of toxoplasmosis

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Summary. - Within its intermediate host, such as man, the protozoan parasite *Toxoplasma gondii* interconverts between tachyzoites and bradyzoites. The replicative tachyzoite stage is thought to be responsible for acute/active infection and expresses immunodominant antigens thereby inducing a strong cellular immune response, which vice versa triggers the differentiation process into dormant and immunologically weak cyst stages. The immunodominance of tachyzoites is also responsible for the induction of a strong humoral immune response leading to the formation of antibodies specifically directed against tachyzoite antigens. In contrast, the bradyzoite stage which is associated with inactive/chronic infection, seems not to be a strong inducer of specific antibodies. However, since the humoral antibody response is also directed against antigens that are expressed in both stages, serodiagnosis cannot always adequately discriminate between active and inactive/chronic infection. This short review focuses on the impact of stage differentiation and discusses the potential of stage-specifically expressed antigens that might be useful in a recombinant form in order to improve future serodiagnostical approaches.

Key words: *Toxoplasma gondii*, diagnosis, persistence, bradyzoite, tachyzoite, recombinant antigens.

Riassunto (*Importanza dello stadio del parassita per la diagnosi della toxoplasmosi*). - All'interno dell'ospite intermedio, quale l'uomo, il protozoo parassita *Toxoplasma gondii* è capace di trasformarsi da tachizoite a bradizoite. Lo stadio di tachizoite è il responsabile della infezione attiva ed acuta ed esprime antigeni immunodominanti inducendo così una forte risposta immune a livello cellulare che invece dà l'avvio al processo di differenziazione verso lo stadio di cisti quiescenti e immunologicamente silenti. L'immunodominanza dei tachizoiti è anche responsabile dell'induzione di una forte risposta immune di tipo umorale che porta alla formazione di anticorpi specificamente diretti contro antigeni di tachizoiti. Al contrario, lo stadio di bradizoite che è associato con l'infezione cronica e inattiva, sembra essere un induttore forte di anticorpi specifici. Comunque poiché la risposta anticorpale è diretta anche verso antigeni che sono espressi in entrambi gli stadi, la sierodiagnosi non sempre può adeguatamente discriminare tra infezione attiva e silente. Questa breve rassegna analizza l'impatto della differenziazione di stadio e discute il potenziale degli antigeni espressi a livello stadio-specifico che potrebbero essere utili, sotto forma ricombinante per migliorare, in futuro, l'approccio sierodiagnostico.

Parole chiave: *Toxoplasma gondii*, diagnosi, persistenza, bradizoite, tachizoite, antigeni ricombinanti.

Introduction

Infection with the protozoan parasite *Toxoplasma gondii* can be caused either by oral ingestion of oocyst-contaminated food or by cyst-harboring raw meat products. Following infection, the parasite undergoes differentiation into the replicative tachyzoite stage which is highly immunogenic and thus induces a cellular-mediated immune response with production of IFN- γ and TNF- α . These cytokines seem to be important for triggering stage differentiation of the parasite from the tachyzoite into the metabolically dormant brady-

zoite stage. In the adult immunocompetent individual, such infection usually does not lead to clinical symptoms. However, bradyzoites persist lifelong within cysts which are primarily located within the brain [1]. One has to differentiate between two important clinical conditions: i) primary infection during pregnancy might cause prenatal toxoplasmosis in the infant, eventually leading to hydrocephalus or retinochorioiditis; ii) immunosuppression of persistently infected patients might cause cerebral toxoplasmosis which is thought to result from cyst reactivation and reverse conversion from bradyzoites back into tachyzoites [2].

Although a tremendous amount of different diagnostic test systems has been developed during the past years, accurate differentiation between acute, latent or reactive infection often is still complicating. This is partially due to the fact that persistent IgM antibodies are found in some patients with past infection, making clear diagnosis difficult especially during pregnancy. Diagnosis of reactivated infection in immunosuppressed patients might be hampered by the low concentration of specific antibodies.

Methods that allow differentiation between active infection and inactive infection should take into account the immunogenicity of the respective parasite stages and the expression of stage-specific antigens. Tachyzoites are replicative stages that are able to destroy the host cell within 48 hours. It is important to note that they are thought to be responsible for active toxoplasmosis and that they express the immunodominant surface antigen SAG1. In contrast, bradyzoites persist in a dormant stage within cysts. These multiparasite structures are thought to evade the immune response by their absence of expression of immunodominant antigens [3]. However, since several diagnostic assays, i.e. Sabin Feldman dye test, are able to detect antibodies even after years of acute infection, it is thinkable that cyst rupture might take place regularly even in immunocompetent patients at low levels allowing individual parasites to re-induce the host's immune response. This review will focus on the differences between tachyzoites and bradyzoites and on the impact that these differences might have on diagnosis of active versus inactive infection.

Bradyzoites and tachyzoites

The development of *in vitro* systems to study bradyzoite-tachyzoite interconversion has opened doors to analyse the precise mechanisms of differentiation. Modifications of the environmental pH, shifting the temperature, IFN- γ treatment, or the inhibition of the mitochondrial respiratory chain induces transition from the tachyzoite to the bradyzoite stage [4]. During this transition, *Toxoplasma gondii* converts its metabolism and its ultrastructural morphology. In contrast to replicative tachyzoites, metabolically dormant bradyzoites are characterized by numerous micronemes, a large number of amylopectin granules, and a nucleus that is located at the posterior end of the parasite [5]. In addition, bradyzoites differ from tachyzoites by the stage-specific expression of certain antigenic components (Table 1). The surface antigens SAG1 and SAG3 are exclusively found on tachyzoites [1]. In contrast, SAG2 has recently been identified to exist as an antigen family which is expressed either on bradyzoites or on tachyzoites, respectively [6]. SAG4

and BSR4/p36 are other surface proteins which are - like the cytosol protein BAG1 - bradyzoite-specifically expressed [7-9].

The fact that external stress and the IFN- γ /NO-mediated inhibition of mitochondrial function induces differentiation into the bradyzoite stage [10] as well as the finding that key enzymes required for the function of both the TCA cycle and oxidative phosphorylation are absent in bradyzoites suggests that this dormant stage relies predominantly on anaerobic glycolysis, whereas tachyzoites can utilize both glycolysis and oxidative phosphorylation [11]. In accordance with this suggestion is the finding that important isoenzymes, which are involved in glycolysis were identified to be stage-specifically expressed. One of these, lactate dehydrogenase (LDH) is a glycolytic enzyme that forms lactate from pyruvate. The two isoforms that are stage-specifically expressed are LDH1 in tachyzoites and LDH2 in bradyzoites. RT-PCR studies demonstrated that *LDH2* mRNA seems to be present only in the bradyzoite stage, whereas transcripts of *LDH1* are present in both stages. However, the LDH1 peptide is only expressed in tachyzoites [12]. Likewise, the enzyme enolase (ENO) is present in two developmentally regulated isoforms. This enzyme catalyses the conversion of 2-phosphoglycerate to phosphoenol pyruvate. In this case, detection of mRNA of *ENO1* was only possible in the bradyzoite stage and *ENO2* transcripts were only found in tachyzoites [13]. Finally, levels of the glycolytic enzyme glucose 6-phosphate isomerase were found to be significantly increased in bradyzoites. This enzyme catalyses the interconversion of glucose 6-phosphate to fructose 6-phosphate [13]. These and other stage-specifically expressed components were identified by the use of expression or subtractive libraries or by comparing the EST transcripts between tachyzoites and bradyzoites [14, 15].

In addition to proteins that are associated with the individual parasite stage, other antigens have been

Table 1. - Stage-specifically expressed antigens of *Toxoplasma gondii*

Stage	Cyst	Membrane	Cytosol
Bradyzoite	CWA MAG1	SAG2C SAG2D SAG4 BSR4/p36	BAG1 ENO1 LDH2
Tachyzoite	-	SAG1 SAG2 SAG2B SAG3	ENO2 LDH1

shown to be exclusively expressed in association with the cyst. One of these is localized to the cyst wall which most likely is generated from the parasitophorous vacuole and which seems to be of parasite origin. The molecular structure of this 115 kDa cyst-wall antigen (CWA) has not been identified, so far [16]. Using a murine monoclonal antibody, Weiss *et al.* [17] identified a 116 kDa antigen which might be related to CWA. MAG1 is an antigen of 65 kDa that has been found within the cyst matrix and to a lesser extent also within the cyst wall [18]. Besides these antigens, microneme, rhoptry, and dense granule proteins were investigated. However, they seem to be expressed in both parasite stages. Additional stage-specifically regulated genes will be identified with the help of recent developments in molecular biology, such as, for example, promoter or gene trap strategies [8, 19].

Since the induction of bradyzoite formation has been suggested to be associated with the presence of external stress and since the expression of heat-shock proteins, such as HSP30 (BAG1), HSP70, and HSP90 is an early event in this differentiation process [1, 20], DNA damage in the cyst stage has been proposed to be a result of such stress events [4]. DNA damage and repair might inhibit parasite replication and thus delays the cell cycle as has been shown in other models [21].

Diagnosis in the immunocompetent individual

In general, diagnosis is based on serological methods. Often, whole parasite cell lysates or SAG1-containing membrane antigens of *Toxoplasma gondii* are used as an antigenic source in order to identify those antibodies that are generated against the tachyzoite stage [22, 23]. Recently, to improve standardization, recombinant SAG1 has been made available for serodiagnosis [24]. However, the western blot technique recently indicated that SAG1 seems not to be a good marker to distinguish acute from chronic infection since corresponding IgG antibodies were determined to have high avidity in both phases of the infection [25].

The conventional Sabin Feldman dye test (DT) is measuring IgG1, IgG3 and IgM antibodies and - although being the golden standard - is now only performed in a few European laboratories. The titer maximum is reached at six to eight weeks after infection and later persists lifelong at low levels, indicating persisting but inactive infection. An immunofluorescence assay (IFA) that is measuring immunoglobulins of all classes usually mimicks the titer kinetics that are found by the DT [26]. The complement fixation test (CFT) likewise is able to

identify the same antibody classes. Since its sensitivity usually is lower than that of the previous two test systems, the titers measured by the CFT are positive later than by using DT or IFA and become negative at an earlier time point after infection.

Test formats that are used to discriminate between IgG, IgM, and IgA antibodies are the ISAGA, ELISA and immunoblot. High-avidity IgG antibodies indicate an infection that usually has been acquired more than four months before [27]. Usually, the combination "IgG positive, IgM and IgA negative" indicates persistent but inactive infection, whereas detection of all antibody classes indicates acute infection. However, there are two exceptions from the rule: i) especially during pregnancy, persistent levels of specific IgM antibodies render it difficult to clearly diagnose acute infection and; ii) in immunocompromised patients, serological diagnosis might be of little help to proof reactivated toxoplasmosis [28].

Diagnosis in the immunocompromised patient

Immunosuppressed patients with reactivated toxoplasmosis often suffer from toxoplasmic encephalitis. Lymphoma is thought to be the most important differential diagnosis in these individuals. Although the detection of specific IgA or/and IgE antibodies is thought to be an indication of reactivated toxoplasmosis [29], serology usually is not so helpful to clearly distinguish between toxoplasmic encephalitis and lymphoma. Likewise, the IgG avidity is also not helpful in diagnosing reactivated toxoplasmosis in immunocompromised patients [30]. In this case, Toxoplasma-specific therapy, such as for example pyrimethamin plus sulfadiazin or clindamycin is started *ex juvantibus* to either confirm or disconfirm the putative diagnosis [28]. Direct detection of the parasite from cerebrospinal fluid or biopsy by using PCR, mouse inoculation or cell culture assays might support diagnosis especially in those patients that are not under prophylaxis or therapy [31]. In this respect, the detection of stage-specifically regulated transcripts would theoretically help to distinguish between acute or chronic infection. However, RNA instability most likely makes this approach not feasible under a practical point of view. Instead, stage-specific monoclonal antibodies that react with parasite antigens under histological tissue-staining conditions are useful tools for differentiating between active infection with tachyzoites or inactive persistent infection with bradyzoites [16]. A combination of such antibodies has demonstrated to be useful for confirming reactivated toxoplasmosis in human tissue samples from AIDS patients [32].

Recombinant antigens for serodiagnosis

Several attempts were made to use recombinant *Toxoplasma gondii* antigens for serodiagnosis. Rojas *et al.* [33] identified HSP90 as a parasite antigen that seems to be helpful for differentiating acute from chronic infection by serodiagnosis: they showed a higher relation of anti-HSP90/anti-*T. gondii* IgG antibodies during chronic infection in comparison to acute infection. When tested, none of the other above mentioned bradyzoite-specifically expressed antigens was shown to be useful for serodiagnosis, so far.

Instead, the tachyzoite-specifically expressed surface molecule SAG1 [34] has been established as a recombinant *Toxoplasma gondii* antigen for routine serodiagnosis that even is commercially available. Another tachyzoite-specifically expressed protein, SAG2 has shown promising results for serologically differentiating acute from chronic infection but this was not followed up later [35]. The rhoptry protein ROP2 is present in both stages of *Toxoplasma gondii* and has been shown earlier to be a candidate antigen for the establishment of serological assays [36]. A recent study indicated that ROP2-specific IgM antibodies have a high correlation with early acute infection [37].

Most scientists agree that a cocktail of recombinant antigens might help to improve serodiagnosis and to replace the tachyzoite antigen SAG1 in serological assays. A combination of recombinant dense granule molecules GRA1 and GRA6 has shown promising results when being preliminary tested [38]. Another example that has successfully been tested in a preliminary formate is the combination of P29, P35, and P66 [39]. Similarly, the combination of recombinant P22/P25P29/P35 confirmed that a cocktail of antigens might be helpful to distinguish between acute and chronic infection when tested for reactivity with specific IgG antibodies in human samples [40].

Discussion

Although our knowledge on stage differentiation of *Toxoplasma gondii* has dramatically increased during the past five to ten years, we are still not knowing the precise factors that promote or prevent this important event in the pathogenesis. Likewise, the identification of a number of stage-specifically expressed antigens has not led to the development of diagnostical test systems that accurately allow the differentiation between acute, chronic, or reactivated infection. However, using serum samples from experimentally infected mice, it has been shown that the serological response to bradyzoite or cyst antigens differ

dramatically from that generated against the tachyzoite stage. The IgG response to cysts was much lower than to tachyzoites, whereas the IgM response developed reversely. This is in contrast to humans, where the IgG and IgM response to cyst antigens was shown to be very low indicating that antigens expressed by this dormant stage seem not to be useful for the development of innovative serological test systems [3].

The use of a combination of recombinant antigens showed promising results being capable to eventually discriminate between acute and chronic infection. It will be a goal for the future to identify those precise antigens that - when used in combination - adequately improve serodiagnosis. Serodiagnosis is of utmost importance since persistence of *Toxoplasma* cysts takes place predominantly in the brain hampering direct detection of the parasite by molecular techniques.

More than ten years after the successful cloning of the SAG1-encoding gene, scientists have developed elegant molecular tools to further dissect the biology of this protozoan parasite but the door to dramatic progress in diagnosis using recombinant antigens has still to be opened. It is therefore hoped that in the future, clinical microbiology will profit from the advances in basic science.

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