



Toxoplasma gondii: Identification and immune response against a group of proteins involved in cellular invasion

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ABSTRACT

Toxoplasma gondii is an ubiquitous intracellular parasite, causative agent of toxoplasmosis, and a world-wide zoonosis for which an effective vaccine is needed. A group of proteins secreted by tachyzoites during host-cell invasion was isolated from the interaction medium. It induced the permeability of the cells as assessed by alpha-sarcin and consequently facilitated the entry of the parasite into the cells. SDS–PAGE of the purified proteins showed a pattern of four proteins of 67, 42, 32 and 27 kDa. MRC-5 cells incubated with the total protein and the different electroeluted bands endured a high cellular death in presence of alpha-sarcin. BALB/C mice immunized with the group of proteins had a mixed Th1/Th2 response and were protected upon challenge with the parasites.

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1. Introduction

Toxoplasma gondii is an obligate Apicomplexan intracellular pathogen that enters both phagocytic and non-phagocytic cells by active penetration (Dobrowolski and Sibley, 1996; Morisaki et al., 1995). It has an extremely broad host range (Dubey and Beattie, 1988) and a unique capacity to invade all nucleated cell types from warm-blooded vertebrate hosts. Toxoplasmosis is one of the major causes of opportunistic infections in AIDS patients and can result in severe damage of newborns during congenital infection. Invasion is an essential step of the pathologies associated with these protozoan parasites that also include *Plasmodium* spp., the causative agents of malaria.

Despite the efficiency of invasion of its host cells by *Toxoplasma* tachyzoites, it is still unresolved whether it occurs by direct penetration or by induction of a host-mediated endocytic event. The chemotherapeutic agents presently used against toxoplasmosis are inadequate, expensive, often toxic and there is still no commercial vaccine for use in humans. A vaccine developed for veterinary use has shown some efficacy (Buxton, 1998). Therefore, the development of an effective vaccine against *T. gondii* would be extremely valuable for preventing both primary fetal infection and reactivation in immunocompromised individuals and might also reduce economic losses by preventing abortions in farm animals.

Although several types of vaccine against toxoplasmosis, which include attenuated and inactivated vaccines, genetically engineering vaccine and DNA vaccine, have been tested for their immunologic effects in animal models, few have been licensed for use, mainly due to poor efficiency or biosafety concerns (Innes and Vermeulen, 2006; Darcy et al., 1992). Inactivated vaccines can only elicit moderate levels of protection (Mishima et al., 2002; Wang et al., 2007). A live attenuated vaccine has been used in some countries, but it is inadequate and expensive, may cause side effects and has the possibility to revert to a pathogenic strain (Bhopale, 2003).

We describe here the isolation of a group of proteins secreted by *T. gondii* tachyzoites during cell invasion, with a capacity to cause the entry of the parasite inside the cells and highly immunogenic to mice.

2. Materials and methods

2.1. Cells and parasites

The RH strain of *T. gondii* was routinely maintained in the human myelomonocytic cell line THP-1 (ECACC 88081201, European Collection of Animal Cell Cultures, Sophia Antipolis, France). The medium used was RPMI 1640 (Sigma–Aldrich, France) supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin (Sigma–Aldrich, L'Isle d'Abeau, France), and 10% fetal calf serum (PAA, les Mureaux, France). The parasites were grown in 150-cm² plastic tissue culture flasks (Falcon, Becton Dickinson, Meylan,

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France). The adherent fibroblasts MRC-5 (CCL-171, ATCC, Manassas, USA) were used for permeability tests.

2.2. Permeability tests

2.2.1. Permeability to alpha-sarcin of *Toxoplasma*-infected cells

To assess the potential capacity of tachyzoites to permeabilize the infected cells, MRC-5 cells were infected at a ratio of 10:1 (parasites/cell) in serum-free medium, and subsequently grown for 12 h. Alpha-sarcin, a 16,800 kDa protein able to block the cellular protein synthesis only if the cells are permeabilized, was then added at final concentrations of 5, 10, 20 and 30 µg/ml. After an additional 4 h-incubation, the cells were washed four times with serum-free RPMI and subsequently cultured in fresh medium with RPMI supplemented with 10% of fetal calf serum at 37 °C for 24 h. The cytopathic effect was assessed using a MTS/PMS solution (CellTiter 96®; Aqueous non-radioactive cell Proliferation Assay, Promega, Madison, WI, USA). Twenty microliters of the solution were added and after 2 h of incubation, the quantity of formazan product as measured by the amount of 490 nm absorbance is directly proportional to the number of living cells in culture.

2.2.2. Permeability of the cells in the presence of secreted proteins

The MRC-5 cells were cultured in presence of the purified proteins (0.0025; 0.005; 0.025; 0.05; 0.1 µg/ml) for 12 h. Then, the cells were incubated for 4 h with alpha-sarcin, the cells were washed four times with serum-free RPMI and subsequently incubated with RPMI supplemented with 10% (v/v) serum at 37 °C. The cytopathic effects of the toxin were evaluated after 24 h using a MTS/PMS solution as it was detailed above.

2.3. Protein isolation and electroelution of the different bands

The released proteins were purified from the medium as follows: the serum was removed from the cells and from the tachyzoites by at least five washes with serum-free medium. The cells were then infected with tachyzoites at a ratio of 1:10 for 16 h. After cell invasion, the infected cells were centrifuged at 1500g, the supernatant was filtered through a 0.2 µm membrane and incubated overnight at 4 °C with 70% ammonium sulfate to precipitate the proteins. After centrifugation at 22000g for 1 h, the protein pellet was resuspended in 0.1 M phosphate buffer, pH 7.1, dialyzed for 72 h and lyophilized. The protein concentration was assessed with a modified Bradford method (RotiNanoquant®, Roth, Brumath, France). The proteins were analyzed with 12% SDS-PAGE gels and visualized either after Coomassie Brilliant Blue or silver staining. The bands of interest were excised from the gel and electroelution was performed using a Hoefer-GE 200, six-Pac gel eluter (Hoefer Scientific Instruments, San Francisco, CA, USA).

2.4. Mice immunization

Eight weeks old BALB/c females mice were purchased from Charles River laboratories (St. Germain sur l'Arbresle, France) and were immunized intraperitoneally (i.p.) with 200 µl of 40 µg of the antigen diluted in NaCl 0.9% containing 2:3 of a commercial adjuvant aluminium gel suspension (13 mg/ml, Sigma-Aldrich). The mice were divided into five groups of ten animals: (1) control healthy mice that did not receive any injection, (2) control adjuvant, mice that received only NaCl 0.9% emulsified in the adjuvant, (3) immunized mice that received the proteins without challenge, (4) immunized infected mice that were infected at day 21 and (5) control infected, naive mice that were infected at day 21 with RH strain tachyzoites. The animals were immunized on day 0 and received a booster injection on days 7 and 14. They were bled on days 0, 7, 14 and 21, and the sera were tested by enzyme-

linked-immunosorbent assay (ELISA) for antibody responses. On day 21, the first group of immunized mice was anaesthetized and sacrificed, their spleen was removed, washed and minced in phosphate buffer saline (PBS). After centrifugation for 10 min at 1000g, the red blood cells were removed by hypotonic lysis for 30 s, the resulting cell suspension was washed in PBS several times and centrifuged. The T-cells were then analyzed by two-color flow cytometry as described below. The sera were stored at –80 °C for cytokine assay. The second group of immunised mice was challenged as described in the next paragraph. All the other animals groups were sacrificed at day 42.

2.5. Challenge infection

Twenty days after the first immunization procedure, mice were intraperitoneally infected with 3×10^6 RH strain tachyzoites. A group of 10 mice was infected at the same time and used as infected control mice.

2.6. Antibody assay

The presence and levels of sera antibodies was analyzed by ELISA in 96-well microplates (Nunc-Maxisorb) coated with 1 µg/ml of the purified proteins in 30 mM carbonate-bicarbonate buffer, pH 9.6. After incubation overnight at 4 °C, the plates were washed with PBS containing 0.05% Tween 20 (PBS-Tween), and blocked with PBS-Tween containing 1% bovine serum albumin for 2 h at 37 °C. Pools of sera diluted at 1/100 in PBS-Tween were added in triplicate wells and the plates incubated at 37 °C for 2 h. After three washes with PBS-Tween, 100 µl/well of diluted specific antibody (1:1000) was added. Antibodies used were goat anti-mouse monoclonals IgG1, IgG2a, IgG2b, IgG3, IgA, IgM (Sigma) IgG (Southern Biotechnology). After incubation for 2 h at 37 °C, the plates were washed, and 100 µl/well of peroxidase conjugated rabbit anti-goat IgG (Sigma) diluted 1:5000 in PBS-Tween was added. After incubation (1 h at 37 °C), Reaction was developed using the substrate 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB, Sigma). Optical densities were read spectrophotometrically at 450 nm with an ELISA plate reader (Microplate Reader, Bio-Rad Laboratories). Sera from twenty 8-week-old normal female BALB/c mice were pooled and used as a negative control to measure the background activity in all experiments. Each assay was performed in triplicate.

2.7. Cytokine assay

The cytokine concentration was determined using commercial sandwich ELISA kits for IL-2, IL-4, IL-12, γ-IFN and IL-6 (Biosource, Nivelles, Belgium). Each assay was performed in triplicate and optical densities were read spectrophotometrically at 450 nm with a plate reader (Microplate Reader, Bio-Rad Laboratories). Sera from twenty 8-week-old normal female BALB/c mice were pooled and used as a negative control.

2.8. Flow cytometric analysis

For immunofluorescence staining with single labeling, 10^6 cells were incubated with FITC-conjugated rat monoclonal antibodies against CD3 (KT3), CD4 (YTS191.1) or with PE-conjugated rat monoclonal antibodies against CD8 (KT15) for 30 min at +4 °C, then washed twice and fixed with 1% paraformaldehyde. Flow cytometric analysis was performed with a Dako Galaxy flow cytometer (Dako, France) equipped with a 488 nm argon laser and detectors for forward scatter (FSC) and 90° light scatter (side scatter, SSC) and for FL1 (band pass filter wavelength 530 nm) and FL2 (585 nm) fluorescence emission in the green part and red/orange part, respectively of the spectrum. Splenocytes stained

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