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Protective Th1 immune responses against chronic toxoplasmosis induced by a protein–protein vaccine combination but not by its DNA–protein counterpart

E. Jongert[a,*,1], D. Verhelst[a,b,1], M. Abady[a,1], E. Petersen[c], N. Gargano[d]

[a] Laboratory for Toxoplasmosis, Pasteur Institute of Brussels, Scientific Institute of Public Health, Brussels, Belgium
[b] Laboratory of Immunology, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium
[c] Department of Infectious Diseases, Aarhus University Hospital, Skejby, Denmark
[d] Kenton Laboratories S.r.l., Rome, Italy

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ABSTRACT

Vaccine-induced protection against toxoplasmosis is correlated with cellular immune responses to *Toxoplasma gondii*, both in animals and man. The goal of the current study was to evaluate whether the combination of a recombinant protein and a plasmid DNA vaccine could offer an advantage over the protein mixture, and protect outbred mice against infection with *T. gondii*. To this purpose, the chimeric protein rEC2, encoding antigenic fragments of surface-associated proteins MIC2, MIC3 and SAG1, was combined with pGRA7 plasmid DNA or rGRA7 protein. High levels of antibodies were elicited by both vaccine formulations. The protein–DNA vaccine elicited a polarized Th1/Th2 immune response, characterized by IFN-γ and IL-10, and afforded low protection (24%) against brain cyst formation. In contrast, the protein–protein vaccine elicited a Th1-focused immune response, characterized by IFN-γ and IL-2 production, conferring a strong protection (79%) against brain cyst formation in chronic toxoplasmosis. We show here that GERBU adjuvanted protein vaccines confer better protection against toxoplasmosis than the protein–DNA heterologous vaccine.

1. Introduction

The protozoan parasite *Toxoplasma gondii* is at the origin of a worldwide zoonosis and it is estimated that one third of the human world population is infected with this parasite. In humans, resolution of the infection occurs through the induction of strong and persistent cell-mediated immunity that results in the control of *T. gondii*, and establish a chronic asymptomatic infection in healthy individuals. The most important group of individuals at risk is pregnant women, in whom a primary infection during pregnancy may lead to infection of the fetus and congenital toxoplasmosis. In contrast, chronically infected women have developed an immune response that prevent transmission to the unborn child. Studies in experimental animal models have shown that the correlate for protection is a strong cellular immune response, with *T. gondii* specific production of IFN-γ and cytotoxic T-lymphocytes as markers for protection [1–4].

The main surface antigen of *T. gondii*, SAG1, is expressed on the surface of free tachyzoites and participates in first contact and attachment of the parasite to the host cell [5]. Upon attachment and gliding, the tachyzoite discharges its micronemes effectively enhancing the strength of attachment of parasite to host cell by the interaction of microneme proteins (MICs) with host cell receptors [6–10]. After internalization, *T. gondii* releases the contents of its dense granules into the newly formed parasitophorous vacuole, and here GRA7 plays an important role in scavenging of nutrients from the host cell [11]. It has been shown that GRA7 is an important vaccine component for optimal protection against chronic toxoplasmosis [1]. Recently, the chimeric protein EC2, encoding antigenic fragments of microneme proteins MIC2 and MIC3 and a fragment of SAG1 was developed for serodiagnosis of human toxoplasmosis, and was successfully recognized by antibodies from mothers infected during pregnancy [12].

The aim of the present study was to investigate whether combined vaccines, based on EC2 and GRA7, could provide a protective activity against *T. gondii*. To this purpose, the EC2 protein was combined with a GRA7 DNA vaccine as a heterologous protein–DNA vaccine mixture and evaluated against the corresponding protein–protein formulation. We show that the heterologous protein–DNA vaccine can induce humoral and cellular immune responses to both vaccine components, and that the

* Corresponding author at: Toxoplasma Vaccinology, Laboratory for Toxoplasmosis, Pasteur Institute of Brussels, Belgium. Tel.: +32 3 373 33 77; fax: +32 2 373 32.81.
E-mail address: ejongert@pasteur.be (E. Jongert).
[1] These Authors contributed equally to this work.

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cellular Th1 immune response contributes in protection against toxoplasmosis.

2. Materials and methods

2.1. Cloning and purification of rEC2 and rGRA7

The chimeric antigen EC2 is made up of fragments of MIC2 (aa 273–345), MIC3 (aa 234–307), and SAG1 (aa 182–312) as described elsewhere [12], contains a total of 24 cysteins and has a predicted molecular mass of 30.1 kDa. The EC2 open reading frame was amplified with primers EC2F (GCGCGGATCCCTCCCCCAGGATGCCATT) and EC2R (GCGCGGATCCAAGCTTCTAGCCGATTTTGCTGACC) by PCR, restricted overnight with BamHI and HindIII endonucleasess and ligated into pQE80 (QIAGEN GmbH, Hilden, Germany). Transformed *E. coli* TOP10F' bacteria were identified on LB/ampicillin agar plates by colony-PCR with the same primers and confirmed by restriction analysis. Positive clones were confirmed by sequencing on a PerklinElmer automated sequencer. A single colony of Top10F' pQE80-EC2 bacteria was grown overnight at 37 °C in LB/ampicillin, and 2 L cultures were inoculated 1/50 with this preculture. Production of histidine-tailed recombinant (r)EC2 was induced with 1 mM IPTG for 2 h at 37 °C. After centrifugation, the bacterial pellet was resuspended and lysed overnight in 100 mM NaH_2PO_4 10 mM Tris–HCl 8 M urea pH 8, followed by 3 freezing–defreezing cycles and sonication until a clear lysate was obtained. After centrifugation for 20′ at 4000 rpm (4 °C) the supernatant was mixed with Ni-NTA Superflow resin (QIAGEN GmbH, Hilden, Germany) for 4 h under gentle rotation at room temperature. The resin was centrifuged 1′ at 1000 × *g* and the sediment was incubated overnight under gentle agitation with wash buffer (100 mM NaH_2PO_4 10 mM Tris–HCl 8 M urea 1 mM 2-β-mercapto-ethanol 0.1% SDS 5 mM imidazol pH 8). The resin was loaded on polypropylene columns with filters calibrated with wash buffer, followed by three alternate washings with wash buffer and wash buffer with 50% isopropanol in order to remove contaminants and endotoxin. Recombinant histailed EC2 (rEC2) was eluted from the resin by gravity flow, after a 2-h incubation in 10 mL elution buffer (100 mM NaH_2PO_4 10 mM Tris–HCl 8 M urea 4 mM 2-β-mercapto-ethanol 0.1% SDS 500 mM imidazol pH 8) and gentle agitation at room temperature. The rEC2 eluate was dialyzed in a 25-kDa cut-off CelluSep H1 membrane (Membrane Filtration Products Inc., Seguin, TX) against 500 mL PBS 0.01% SDS 1 mM 2-β-mercapto-ethanol 2 M urea ON, followed by dialysis against 500 mL PBS 0.01% SDS 0.1 mM 2-β-mercapto-ethanol 1 M urea for 2 h, against 500 mL PBS 0.01% SDS 0.01 mM 2-β-mercapto-ethanol 0.5 M urea for 1 h, and finally against 500 mL PBS 0.01% SDS 0.01 mM 2-β-mercapto-ethanol 0.1 M urea for 1 h.

Histidine-tailed rGRA7 protein, pGRA7 plasmid DNA and empty vector VR1020 were produced as described previously [1].

2.2. Vaccine formulation and immunization of mice

Swiss F1 outbred female mice, aged 6 weeks were obtained from the breeding facility of the Pasteur Institute of Brussels, and housed in line with guidelines from the local animal committee. Vaccines were formulated as described in Table 1. Recombinant EC2 was combined with rGRA7 protein or pGRA7 DNA, in GERBU; an adjuvant based on cationic lipid solid nanoparticles and *N*-acetylglucosaminyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine, a glycopeptide derived from *Lactobacillus bulgaricus* cell walls [13] (GERBU Biochemicals GmbH, Gaiberg, Germany). Mice received from 7 weeks of age onwards two injections of rEC2 + pGRA7, rEC2 + rGRA7, or empty vector (VR1020), separated by a 3 weeks interval. The vaccine was administered intramuscularly with a 0.3-ml syringe and the volume was divided over both tibialis anterior muscles. To obtain chronically infected mice, Swiss mice were fed a sublethal dose of 20 brain cysts of *T. gondii* 76 K.

Table 1
Vaccine formulations

Antigens	Vaccine formulations		
	Empty vector	rEC2 + rGRA7	rEC2 + pGRA7
rEC2	–	10 μg	10 μg
rGRA7	–	10 μg	–
pGRA7	–	–	50 μg
VR1020	50 μg	50 μg	–
GERBU Adjuvant	50 μl	50 μL	50 μL
Total volume/shot	100 μL	100 μL	100 μL
Total DNA/shot	50 μg	50 μg	50 μg
Total protein/shot	–	20 μg	10 μg

2.3. Antibody enzyme-linked immunosorbent assay (ELISA)

Prior to vaccination and 3 weeks after every injection, mice were bled from the orbital plexus and sera were analysed by ELISA to measure antigen-specific IgG1 and IgG2a antibodies. Maxisorp ELISA plates (Roskild, Denmark) were coated overnight with rGRA7 or rEC2 at 10 μg/mL in 50 mM bicarbonate buffer (pH 9.6) at 4 °C. ELISA was performed as described previously [14]. Sera were applied in twofold dilution series, starting at a dilution of 1/50. Endpoint titers were defined as the dilution where the optical density (OD_{450}) exceeded the cut-off value. Cut-off was calculated as the mean OD_{450} + three times the standard deviation (S.D.), from sera from control-vaccinated mice at a dilution of 1/50.

2.4. Indirect immunofluorescence assay (IIFA)

Recognition of parasites by antibodies was evaluated by indirect immunofluorescence assay. Fifty microliters of pooled sera diluted 1/50 in PBS was applied on slides coated with formalin-treated tachyzoites from the RH strain (Toxo-Spot IF, Bio-Mérieux, Marcy-l'Etoile, France) and put for 30 min in a 37 °C incubator. Slides were washed with PBS, water and dried for 5–10 min at 37 °C. Slides were incubated 30 min at 37 °C with 30 μL of Alexa Fluor 488 conjugated goat-anti-mouse IgG (Invitrogen, Merelbeke, Belgium) diluted 1/500 in PBS with Evans Blue (1/100,000). After washing and drying, the slides were read with a fluorescence microscope (Carl Zeiss). The cut-off read-out of the fluorescence test was established at a dilution of 1/40 with both toxoplasma seronegative and seropositive murine reference sera from our laboratory. The specificity and sensitivity of the Toxo-Spot IF test for IgG are 98.44 and 95.08%, respectively.

2.5. Western blot

Antibody detection of parasite components was evaluated by Western blotting as described previously [15]. Briefly, total lysate antigen (TLA) of *T. gondii* RH, or glutathione *S*-transferase (GST) linked $MIC2_{273–345}$, $MIC3_{234–307}$, and $SAG1_{182–312}$ fragments [12,16] were separated by SDS-PAGE and transferred to a nitrocellulose membrane (BioRad Life Sciences, Nazareth, Belgium) by Western blotting. After blocking, pooled sera from the vaccinated and infected mice was incubated 1/500 in PBS 10% FCS, overnight at 4 °C with gentle agitation. After incubation with a secondary horse radish peroxidase conjugated anti-mouse IgG antibodies (AbD Serotec, Oxford, UK), protein bands were visualized by development with 4-chloro-napthol substrate (Sigma, St. Louis, MO). The reaction was stopped by rinsing with tap water.