Review Article

Toxoplasma Rhoptries: Unique Secretory Organelles and Source of Promising Vaccine Proteins for Immunoprevention of Toxoplasmosis

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Toxoplasma gondii is an obligate intracellular protozoan parasite classified in the phylum Apicomplexa, which includes numerous notable human and animal pathogens (*Plasmodium* species, *Cryptosporidium* species, *Neospora caninum*, etc.). The invasive stages of apicomplexans are characterized by the presence of an apical complex composed of specialized cytoskeletal and secretory organelles, including rhoptries. Rhoptries, unique apical secretory organelles shared exclusively by all apicomplexan parasites, are known to be involved in an active parasite's penetration into the host cell associated with the biogenesis of specific intracellular compartment, parasitophorous vacuole in which the parasite multiplies intensively, avoiding intracellular killing. Due to the key biological role of rhoptries, rhoptry proteins have recently become vaccine candidates for the prevention of several parasitoses, toxoplasmosis among them. The article presents current data on *T. gondii* rhoptries biology and new approaches to the development of effective vaccines against toxoplasmosis using rhoptry antigens.

1. INTRODUCTION

Toxoplasma gondii is arguably the most successful protozoan parasite on earth. It infects humans and many species of endothermic animals with a great infection rate, achieving nearly 100% in some local populations [1]. The course, symptoms, and consequences of *T. gondii* infection depend strongly on the virulence and inoculum size of the parasite and the genetic background and immune status of the infected host. In general, most postnatal cases of toxoplasmosis are mild and subclinical, with lifelong persistence of the parasite in the host. However, in immunosuppressed beings, both primary infection and reactivation of chronic infection could occur very serious, causing even death. Primary infection acquired during pregnancy can lead to an abortion and neonatal malformations. Unfortunately, despite many efforts there are no satisfactory immunoprevention methods against toxoplasmosis. The only accepted vaccine against toxoplasmosis (Toxovax, Ovilis Toxovac) contains live tachyzoites of incomplete nonpersistent S48 strain and is used to prevent congenital toxoplasmosis in sheep. The vaccine causes a decrease in abortion cases frequency but not complete eradication of the parasite [2].

2. *TOXOPLASMA GONDII*: OBLIGATE INTRACELLULAR APICOMPLEXAN PARASITE

Successful parasitism by *T. gondii* is based on its ability to form within the host cell a replication permissive niche—parasitophorous vacuole (PV). This growing niche is delimited from the host cell cytoplasm by PV membrane (PVM), a unique and dynamic "organelle" found only in infected cells which prevents *T. gondii* degradation by the host cell endocytic machinery and enables parasite's intracellular propagation. Unique to the apicomplexan protozoa including *Toxoplasma gondii* are also three types of electro-dense secretory organelles: micronemes, rhoptries, and dense granules, carrying their characteristic proteins: MICs, RONs, ROPs, and GRAs, respectively; see Table 1. Host cell invasion is mediated by the sequential secretion of the contents of all three organelles, which are exocytosed at the apical region when the parasite invades the host cell. The processes,

TABLE 1: The participation of secretory organelles proteins in the invasion of the host cell by protozoan *Toxoplasma gondii*; PV: parasitophorous vacuole, MICs: micronemes proteins, TgAMA1: *T. gondii* apical membrane antigen 1, RONs: rhoptry neck proteins, ROPs: rhoptry proteins, GRAs: dense granule proteins.

Invasion stage	Secretory organelles/*secretion process* secreted proteins
Adhesion	Micronemes/*transient* TgAMA1,TgMICs etc.
Biogenesis of moving junction, PV and its association with host mitochondria and endoplasmic reticulum	Rhoptries/*transient* RONs, ROPs
Formation of the specific architecture and function of PV	Dense granules/*continuous* GRAs

critical in the establishment of a productive infection, are highly regulated at possibly two levels: physical separation of exocytosis sites in the parasite's plasma membrane and different trigger mechanisms [3, 4]. Micronemes are involved in the attachment and penetration of *T. gondii*, while rhoptries are required for creating a transient enigmatic structure, moving junction and then the establishment of PV. Host cell entry of the parasite is completed in 15–20 seconds [5, 6]. Dense granules secrete proteins throughout most of the parasite stages. The secretion process coincides with the formation of intravacuolar network and continues during the intracellular residence of *T. gondii* [3, 4].

3. RHOPTRIES AND RHOPTRIES PROTEINS

Unlike most secretory and lysosomal granules in mammalian cells, which are of a semispheroidal shape, the mature rhoptries are club shaped with a bulbous base and an extended duct (neck), connected to the extreme apical pole of the parasite. The rhoptries in the number of 8–12 per cell occupy 10–30% of the total cell volume. They are the only known acidified organelles in *T. gondii*; pH of immature rhoptries is 3.5–5.5 and of mature rhoptries is 5.0–7.0 [7]. Most probably each mature rhoptry originates from a separate immature rhoptry [8].

Current findings suggest that rhoptries are most analogous to secretory lysosomal granules because they receive material from both biosynthetic and endocytic cell pathways. In the exit site of endoplasmic reticulum, the rhoptry proteins (in a form of proproteins) are loaded into coated vesicles and then travel to the Golgi apparatus where they are sorted to an immature rhoptry using the specific sorting signals [9]. Both tyrosine-based and dileucine sorting motifs were detected within cytoplasmic tails of the predominant ROP2 family proteins [10]. Proproteins undergo proteolytic cleavage of an N-terminal prodomain, which occurs in post-Golgi compartment, probably in immature rhoptries [11]. In contrast to all known prominent ROP2 family members, a new described ROP5 protein is not processed during trafficking but is synthesized in the mature form [12]. Some rhoptry cargo is delivered by endosomal pathway via multivesicular body and immature rhoptry [9]. Immature rhoptries evolve into mature rhoptries characterized by

specific morphology and subcompartmentalization of their contents: ROP proteins are located in the bulb body whereas RONs in the neck of the rhoptry [13].

A proteomic analysis revealed over 30 rhoptry proteins, all localized in the contents of the rhoptries, although many of the identified proteins have putative transmembrane domains [13]. As shown for ROP2, a prototype protein of ROP2 protein family, apart from an N-terminal signal sequence, the molecule is characterized by the high abundance of charged amino acids and proline residues, N-terminal arginine-rich stretches and hydrophobic sequence in C-terminus considered as transmembrane domain [14, 15]. The ROP2 protein was shown to be inserted in PVM during the invasion of a host cell and its N-terminal domain is exposed to host cytosol [14] mediating the association of parasitophorous vacuole with host cell mitochondria [14, 16]. Targeted depletion of ROP2 resulted in multiple effects, such as: (i) impairments of rhoptry biogenesis and cytokinesis, (ii) reduction in the association of host cell mitochondria with PVM of the parasite, and a reduced sterol uptake from the host cell, and (iii) reduced capacity to invade and replicate in vitro in human fibroblasts and attenuation of virulence in mice [17]. Besides, anti-ROP2 antibodies caused a decrease in the invasion ability of *T. gondii* RH tachyzoites in vitro [18]. The observed effects of ROP2 depletion or inactivation and simultaneous synthesis of several related proteins (ROP2 family) suggest that they serve crucial biological functions. All members of the ROP2 family contain a protein kinase-like domain but only some of them are catalytically active [15]. The exocytosis of rhoptry contents coincides with the formation of PV but the biological trigger of rhoptry secretion has not been identified. Mobilization of intracellular Ca^{2+} by ionophores triggers the discharge of micronemes but not rhoptries and dense granules [19]. In contrast to other secretory organelles, rhoptry proteins and lipids are exocytosed through the duct, whereas delimiting membrane is retained in the cell and the rhoptry is transiently empty. Secreted rhoptry proteins are subsequently discharged into nascent parasitophorous vacuole and localized in its membrane [14, 20].

As mentioned earlier, certain rhoptry proteins are preferentially localized in the neck of the rhoptry. During invasion, RON4 relocalizes at the moving junction and participates

in the formation of this short living and very enigmatic structure between the host cell and the parasite, sliding over the parasite as it invades [21]. RON4, RON2, and RON5 (Ts4705) form protein trio that associates with adhesion microneme protein TgAMA1 (*T. gondii* apical membrane protein 1) forming an unusual complex derived from two distinct secretory organelles: rhoptries and micronemes [22]. Moving junction functions as a molecular sieve for the quick selective sorting of parasite surface components (like RONs-TgAMA1 complex mentioned above) as well as surface proteins of the host cell. Because of the exclusion of most host cell membrane proteins, developing parasitophorous vacuole is not recognized by the host cell fusogenic machinery enabling the vigorous parasite proliferation in this safe intracellular compartment [5, 23].

4. VIRULENCE OF *T. GONDII* AND NEW DESCRIBED RHOPTRY ANTIGENS AS MAJOR VIRULENCE COMPONENTS

Despite the very high genetic homology achieving ~98%, *T. gondii* strains express dramatic differences in many phenotype aspects, including virulence. The majority of *T. gondii* isolates from Europe and North America belong to three clonal lines (types) significantly differing in their virulence, which has been well characterized in the mouse model. Type I isolates have an LD100 of a single organism and lead to rapid death of the infected mice (6–10 days), whereas types II and III (called nonvirulent or avirulent) typically have LD100 of $\geq 10^3$ organisms; mice which survive acute phase of toxoplasmosis remain chronically infected and highly seropositive [24].

Dramatic differences in acute virulence between three major types of *T. gondii* enabled the mapping of virulence genes by an analysis of F1 progeny from genetic crosses between three toxoplasma types, using an experimental mouse model to test virulence. The method applied to types I × III recombinants revealed first two closely adjacent virulence loci on parasite chromosome VIIa and then single gene encoding ROP18 protein as a major virulence component. Type III and type I alleles of ROP18 differed significantly in nucleotide sequence and expression level. The contribution of ROP18 to virulence was confirmed by the transfection of the virulent ROP18 allele into nonpathogenic type III parasite strain. The obtained transformants showed an increased growth rate in vitro and an enhanced mortality by 4 to 5 logs in CD1 mice, as compared to wild nontransformed strain [25]. Saeij et al. [26] mapped virulence in F1 progeny derived from crosses between type II and type III strains. Five virulence loci were identified, and two of them coded rhoptry proteins: ROP18 and ROP16, both hypervariable protein kinases that are responsible for the high virulence of certain strains of the parasite. Contrary to ROP2 and several other members of this family that lost detectable kinase activity [15] and retained "molecular fossils" [27], newly described rhoptry proteins ROP16 and ROP18 are true kinases, the substrates of which are unknown. Both kinases presumably disrupt some signaling processes and alter intracellular environment in a way that favors parasite

growth. The work of El Hajj et al. [28] brought many interesting data on ROP18. The protein showed a very strong tropism to PVM and during the invasion it was translocated from rhoptry to PVM. The mature recombinant ROP18 was able to phosphorylate two as yet unknown parasite's (but not host's) proteins, a major one of 70 kDa and a minor one of 68 kDa. The overexpression of ROP18 protein promoted dramatically parasite's proliferation, whereas the mutation (D394A) in ROP18 molecule resulted in the loss of both the enzyme activity and accelerated growth. In contrast to ROP18, the second recently described rhoptry protein, ROP16 is translocated to the host cell nucleus like phosphatase 2C [29], subverts STAT3/6 signaling, and, in consequence, IL-12 production in infected host cells. Allele shared by types I and III of the parasite induce prolonged phosphorylation of STAT3 (STAT3-PO$_4$) which results in a much lower level of IL-12 secretion, as compared to type II allele [30]. The observed type-specific effects show the key role of ROP kinases in *T. gondii* virulence and could explain different disease outcomes seen with type I, II, and III strains. The role of ROPs in virulence suggests that they could be promising vaccine candidates against toxoplasmosis.

In contrast to ROP18 and ROP16, another novel rhoptry protein, bradyzoite rhoptry protein 1 (BRP1), is expressed in bradyzoites but absent from tachyzoites. Bradyzoites are a low-replicating, inactive developmental stage, sequestered in tissue cysts, which are a typical marker of chronic toxoplasmosis. The biological function of BRP1 is not defined yet. This protein does not play an essential role in the development of the bradyzoite stage, brain cyst formation, and oral infection of new hosts [31].

5. IMMUNOPROPHYLAXIS OF TOXOPLASMOSIS

As mentioned earlier, the only vaccine against toxoplasmosis has been licensed for use in sheep in Europe and New Zealand [2]. This conventional vaccine contains live attenuated tachyzoites of the nonpersistent *T. gondii* S48 strain and it cannot be considered for an application in humans because of the risk of reverting to tissue cyst formation. From both humanitarian and economic points of views, an effective anti-*T. gondii* vaccine(s) for humans and animals is still strongly desired. Taking into account the main sources and transmission routes of *T. gondii*, host groups of a particularly high infection risk and consequences of congenital infection, as well as postnatal infections in immunocompromised individuals, targets for vaccination strategy should include (i) preventing tissue cyst formation in consumption animals (to avoid parasite transmission to humans and animals) and in people (to protect against reactivation of infection in immunosuppression state of persistently infected individuals), (ii) reducing oocyst shedding in cats (to limit environmental contamination and infection risk for all intermediate hosts), and (iii) preventing development of parasitemia in pregnant women and farm livestock (to avoid transplacental transmission to the fetus and congenital toxoplasmosis) [32].

Vaccine candidates should induce protective cellular TH1 and humoral responses as well, both at the level of intestinal

mucosa (local) and whole organism (systemic). Many studies have shown that protective immunity to *T. gondii* is characterized by the development of cell-mediated immunity dominated by the production of IFN-γ by T lymphocytes (a TH1 type response). Whereas immune CD8+ lymphocytes are cytotoxic for *T. gondii*-infected host cells, CD4+ lymphocytes exert their protective activity primarily by the production of many proinflammatory cytokines (IFN-γ, TNF-α, IL-12) and providing growth factor, IL-2. Specific antibodies limit multiplication of *T. gondii* by killing of extracellular tachyzoites, either by activating complement, or opsonizing the parasites for phagocytosis and next killing by macrophages [33]. The major challenge in novel vaccine construction is a selection of relevant *T. gondii* antigens and then their presentation to host immune system in an appropriate manner to induce strong and long-lasting protective immunity. Because of the complex life cycle of the parasite, the expression of numerous common and stage-dependent antigenic epitopes and host-dependent immune responses, the vaccination with multiantigen preparation comprising antigens from different life cycle stages is likely to be more efficacious than with a single antigen. Previous and current vaccine approaches focus not only on classic formulations (live attenuated or mutant *T. gondii* strains) [2, 34, 35] but more often on the defined subcellular components of the parasite such as biosynthetic (recombinant) antigens [36] and naked *T. gondii* DNA [37] or RNA [38]. Nonpathogenic recombinant microorganisms with the expression of immunoprotective antigens of *T. gondii* (live vector vaccines) [39] and synthetic peptides have also been tested as interesting antitoxoplasmic vaccine alternatives [40]. These new generation subunit vaccines require an addition of potent adjuvants enhancing the intensity of the immune response and inducing the desired TH1 profile. Several new compounds, for instance unmetylated CpG nucleotides, present satisfactory adjuvant TH1 activity [41].

6. RHOPTRY ANTIGENS AS A POTENTIAL VACCINE AGAINST TOXOPLASMOSIS

Most of the current vaccine candidates for *T. gondii* and other apicomplexa are either surface or secreted antigens that appear to be essential for the invasion process, rhoptry proteins among them. Until now several rhoptry proteins of *T. gondii* have been tested as vaccines. Garcia et al. [42] used crude native rhoptry antigens incorporated in the immunostimulating complexes (ISCOMs) to protect pigs against an infection with oocysts of *T. gondii* VEG strain (of type III). Two-fold subcutaneous immunization procedure led to a partial protection against cysts formation in muscles and brain during chronic infection (as confirmed by bioassay in mice) but it was not protective against acute infection, probably because of the lack of intestinal immunity after subcutaneous immunization. Taking into account epidemiological role of cats as an important source of *T. gondii* dissemination by shedding millions of oocysts in feces, the authors tested the same antigen material in domestic cats [43]. The cats were immunized intranasally three times (0, 21, 42 day) with crude rhoptry proteins adjuvanted with

Quil-A and then challenged with tissue cysts of the *T. gondii* VEG strain. This vaccination procedure resulted in a partial protection only—two from three immunized cats did not shed oocysts.

Till now vaccine trials with individual ROPs of *T. gondii* have mostly focused on the ROP2 antigen, prominent member of ROP2 protein family. In majority, the experiments were performed with DNA vaccines which are believed to elicit a predominant TH1 response. Vercammen [44] showed that the immunization with ROP2 gene protected mice against lethal challenge with a highly virulent RH strain, but the observed protection was mouse strain dependent— inbred C3H mice were protected, whereas C57BL/6 and BALB/c were not.

Similar results were obtained by Leyva et al. [45] in mice of three inbred strains (CBA/J, C57BL/6, and BALB/c), vaccinated with plasmid encoding antigen ROP2. The mice did not resist a challenge with RH strain but the protection was achieved by vaccination with live tachyzoites of the *T. gondii* nonpersistent thermosensitive ts-4 strain. In the mice immunized with ROP2-plasmid, a mixed TH1/TH2 immune profile instead of a desirable TH1 profile and a significant variability in the response between individual mice was observed. Using similar vaccine material, that is, ROP2-plasmid, Wei et al. [46] reported both a high immune response and significant immunoprotection against a lethal dose of *T. gondii* RH strain in BALB/c mice.

Recently, live nonpathogenic microbes (viruses or bacteria) were used as vectors of selected ROP genes. Recombinant vaccinia virus carrying ROP2 gene induced in mice a specific humoral response very similar, in intensity and profile, to the thermosensitive ts-4 strain of *T. gondii*. Because the ts-4 strain as vaccine was able to fully protect mice against the challenge with the highly virulent RH strain, the authors conclude by analogy that ROP2-vaccinia virus could be equally effective in toxoplasmosis immunoprotection [47]. Mishima et al. [18] obtained recombinant feline herpesvirus 1 (FHV1) encoding ROP2 and evaluated its ability to induce antitoxoplasmic activity and protective immune responses. Antisera of ROP2-FHV1-immunized cats reduced significantly the invasion capacity of *T. gondii* RH tachyzoites in vitro. The number of parasites in the brains of immunized cats lowered, however oocyst shedding intensity was not reduced. Recently, Wang et al. [48] tested the efficacy of recombinant *Mycobacterium bovis* BCG expressing ROP2 gene as a vaccine, using mouse experimental toxoplasmosis model. BCG, widely applied in immunoprophylaxis of tuberculosis, is a particularly attractive vector for delivering heterologous antigens because mycobacteria elicit TH1-mediated immune response without an additional adjuvant [49]. Vaccine ROP2-BCG induced strong specific humoral and cellular (IL-2 and IFN-γ in supernatants of splenocytes cell cultures) immunity. A significant delay in mortality after the infection with the highly virulent RH strain was also observed [48].

The use of recombinant ROP2 antigen, instead of ROP2-plasmid, revealed again in mouse experimental model that the protective activity of ROP2 protein is mouse strain-dependent; ROP2 vaccination resulted in the partial

protection (decrease in tissue cyst burden) only in C3H mice but not C57BL/6 mice [50]. The efficacy of ROP2 protein vaccine significantly increased by including new immunostimulatory components, *Leishmania infantum* heat shock protein 83 (Hsp83) fused with the ROP2 [51]. Immunization with this fusion protein elicited a predominant TH1 response in all mouse strains used (BALB/c, C57Bl/6, and C3H), whereas ROP2 alone or in mixture with Hsp83 induced a mixed TH1/TH2 profile.

Multiple endogenous and exogenous parameters can influence the type and intensity of vaccine-induced immune response. The results obtained on inbred mouse strains proved that the protection induced by ROP2 and other vaccine antigens of *T. gondii* is strongly dependent on the host genetic background [43, 50, 52]. In relation to the vaccine itself, the composition seems a very important factor in the context of both selected ROP antigens and adjuvants used. In the light of our current knowledge on rhoptry antigens, ROP18 protein, described recently as a major component of pathogenicity of *T. gondii*, seems a very promising candidate for a vaccine, as a single antigen or, more possibly, accompanied by other *T. gondii* antigens because many previous studies showed greater effectiveness of combined than single-antigen vaccines. However, the usefulness of ROP18 as a vaccine antigen for toxoplasmosis needs to be experimentally confirmed, especially in relation to the polymorphism of this protein. Whether protection across *T. gondii* species could be achieved with that polymorphic antigen remains an open question.

The advantages of combined vaccines as compared to single-antigen vaccines were recently emphasized by Zhang et al. [53] who constructed a multiantigenic DNA vaccine consisting of plasmid encoding both ROP2 and surface antigen 1 (SAG1) antigens and inoculated it to BALB/c mice with or without plasmid encoding murine IL-12. Multiantigenic (cocktail) vaccine elicited stronger antibody and cellular responses than single-gene vaccines and, additionally, coimmunization with IL-12 gene significantly enhanced the intensity of immune responses. The most immunostimulating vaccine, pSAG1+ROP2+pIL-12, delayed about three times the survival time of mice after a lethal challenge with RH tachyzoites. As found by Guo et al. [54] another genetic adjuvant, plasmid carrying IFN-γ, revealed also a potent stimulatory activity, enhancing the cellular immune response induced in mice by ROP1-plasmid.

In summary, all the above presented experimental vaccination strategies with ROP antigens still remain suboptimal but the vaccination results appear promising and therefore future research on ROP vaccine seems rational.

7. CONCLUDING REMARKS

Toxoplasmosis, widely dispersed in the populations of humans and endothermic animals all over the world, is a serious problem of human and veterinary medicine in the case of immunocompromised individuals (fetuses, AIDS patients, etc.). The consequences of primary infection in pregnant females and the possibility of reactivation of latent infection in immunodepressed individuals cause that both immunoprophylactic and immunotherapeutic vaccines are needed. Natural infection with *Toxoplasma* leads to strong, lifelong nonsterile protective immunity. The persistence of the memory T lymphocytes, stimulated by the regular rupture of *T. gondii* tissue cysts or by recurrent contact with infected food, prevents a reinfection in most individuals [33]. The effective and safe vaccine should mimic the immune response observed in natural infections and protect the hosts against *T. gondii* infection, however without the potential risk of tissue cyst generation in intermediate hosts and development of oocysts in cats, associated with the vaccination using live attenuated or incomplete parasite strains. In this context, for *T. gondii* ROP antigens as key molecules in biogenesis of parasite intracellular niche in the host cells and their association with host organelles, major virulence factors showing satisfactory immunogenicity [55] seem particularly valuable vaccine candidates for toxoplasmosis prophylactic approaches but their vaccine potential needs experimental confirmation. It is worth mentioning that rhoptry proteins are also vaccine candidates in other apicomplexa, including rhoptry-associated protein 1 (RAP1) and RAP2 proteins of *Plasmodium falciparum* [56]. Several rhoptry proteins are polymorphic, with T and B epitopes that are conserved among strains but not across species and over species, as found for instance for *Plasmodium* [57] and *Eimeria* [58]. The question, if it would be possible to construct universal anti-*T. gondii* vaccine, independent of the host and vaccination target, remains open. The results of the studies on ROP-vaccine presented in this paper are promising and could be a good starting point to develop an effective vaccine not only for the prevention of toxoplasmosis but also other parasitoses caused by apicomplexan protozoa. Recent advances in bioinformatics and experimental tools will make the selection of vaccine candidates easier.

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