Toxoplasmosis is a globally distributed foodborne zoonosis caused by the protozoan parasite Toxoplasma gondii. Usually asymptomatic in immunocompetent humans, toxoplasmosis is a serious clinical and veterinary problem often leading to lethal damage in an infected host. In order to overcome the exceptionally strong clinical and socio-economic impact of Toxoplasma infection, the construction of an effective vaccine inducing full immunoprotection against the parasite is an urgent issue. In the last two decades many live attenuated, subunit and DNA-based vaccines against toxoplasmosis have been studied, however only partial protection conferred by vaccination against chronic as well as acute infection has been achieved. Among various immunization strategies, no viable subunit vaccines based on recombinant secretory (ROP2, ROP4 and GRA4) and surface (SAG1) T. gondii proteins have been found as attractive tools for further studies. This is due to their high, but still partial, protective efficacy correlated with the induction of cellular and humoral immune responses.

Introduction

Apicomplexan protozoan Toxoplasma gondii is one of the most world-wide spread parasites and etiological agent’s of toxoplasmosis. This evolutionarily successful obligate intracellular pathogen invades many species of warm-blooded animals including humans and replicates in virtually all of the hosts nucleated cells. It is estimated that about one-third of the human world population (between 10% and 85% depending on the geographical location) is infected with T. gondii and the foodborne toxoplasmosis is ranked at the same level as salmonellosis or campylobacteriosis. The life-cycle of this intracellular parasite includes definitive and intermediate hosts and three infectious life-stages (sporozoites, tachyzoites and bradyzoites) (Fig. 1). Development of sporozoites is the outcome of the sexual phase of the Toxoplasma life-cycle occurring within gut epithelial cells of Felids, the only known Toxoplasma definitive hosts (e.g., domestic cats). The sexual phase results in the production and shedding of environmentally resistant oocysts in the feces of the infected cats. In contact to the environment, oocysts sporulate and become fully infectious. Finally, a mature oocyst contains two sets of four sporozoites, each set enclosed within a single sporocyst, and is able to infect other definitive or intermediate hosts. Generation of the two other Toxoplasma infectious life-stages, tachyzoites and bradyzoites, takes place during the asexual development in intermediate hosts. Although, the asexual division also occurs in definitive hosts, it is necessary only to increase the number of parasites before the sexual phase of the life-cycle and leads to the meront stage. In intermediate hosts the presence of tachyzoites and bradyzoites depends on the phase of toxoplasmosis, acute or chronic, respectively. After ingestion of oocysts or tissue cysts present in contaminated food, the cysts are ruptured and sporozoites and bradyzoites are released in the intestinal lumen. Both forms infect...
The lifelong protection developed in response to primary Toxoplasma infection indicates that immunoprophylaxis of toxoplasmosis is a realistic goal. In the past several years, many vaccination trials of human and veterinary live attenuated, subunit and DNA-based vaccines have been performed and various levels of protective immunity have been achieved. However, due to the restrictive requirements for human vaccines, it would be rather undesirable to use live attenuated Toxoplasma strains for immunoprophylactic treatment in people.12,13

Protective Efficacy of Recombinant ROP2, ROP4, GRA4 and SAG1 Protein Cocktails

A key factor determining the effectiveness of each vaccine is its antigen composition. Antigens used as vaccination tools should play an important role in pathogenicity and elicit in immunized individuals immune mechanisms required for long-term protection against a desired pathogen. In search of such antigens, the components of the future vaccine for toxoplasmosis, we initially focused on the main pathogenic form.11,12

The importance of B cells for an effective prevention of toxoplasmosis is still not fully understood but their cooperative response seems to be favorable in controlling T. gondii invasion. A synthesis and release of the parasite-specific antibodies could limit Toxoplasma spread by inhibiting the attachment of tachyzoites to host cells and promoting extracellular and intracellular killing of antibody-coated parasites by a complement-dependent pathway and macrophages, respectively.17,19

A substantial clinical problem is also congenital toxoplasmosis resulting from the vertical transplacental transmission of the parasite from a primary infected pregnant mother to the fetus. In congenitally infected infants parasitemia can be manifested as retinochoroiditis, intracranial calcification, microcephaly, hydrocephalus, mental retardation or can even result in spontaneous abortion and neonatal death. In veterinary medicine infections with T. gondii in free-ranging farm animals have economic importance due to stillbirth, abortion and neonatal loss in many kinds of livestock. Moreover, similarly to oocysts contaminating the environment (e.g., water, soil, vegetables and fruit), the parasite tissue cysts contained in meat or meat products from infected livestock, mainly poultry and pigs, are considered as a major source of the parasite's horizontal transmission to humans.1,5,18

Efforts to overcome an enormous clinical and socio-economic impact of toxoplasmosis have been focused mainly on the development of efficient tools for immunoprophylaxis of this disease. The available anti-parasitic chemotherapeutic agents used for the treatment of Toxoplasma infections (e.g., pyrimethamine, sulfadiazine) fail to eliminate the lifelong chronic infection and frequently cause adverse toxic effects. Currently, the only commercially available vaccine against toxoplasmosis is “Toxovax” based on live tachyzoites of T. gondii S48 strain attenuated by repeated passages in mice. This vaccine is mostly used for sheep immunization to diminish the economic losses related to the parasite-induced abortion. Unfortunately, vaccination with “Toxovax” is not approved in humans due to the short-lived infection induced by S48 tachyzoites and a possible risk of the reversion of the attenuated mutant into the pathogenic form.15,17

Globally distributed infections with T. gondii represent a serious clinical and veterinary problem. In immunocompetent individuals toxoplasmosis is mostly asymptomatic or could be demonstrated by nonspecific mild mononucleosis-like symptoms manifested predominantly as lymphadenopathy. A real clinical problem is posed by devastating T. gondii invasions in immunocompromised persons such as AIDS patients, organ transplant recipients and patients with malignancies leading even to lethal damage in infected hosts. Primary adult acquired infection with T. gondii in immunocompetent hosts induces strong cellular and humoral immunological responses resulting in both lifelong protection and lifelong chronic infection. The protective immunity rests mainly on innate and adaptive cellular mechanisms. The crucial element of innate cellular immunity is intereleukin 12 (IL-12) produced by dendritic cells, macrophages, monocytes and neutrophils. This cytokine is responsible for the regulation of IFN-γ synthesis by natural killer (NK) cells as well as by Th1 CD4+ and cytotoxic CD8+ lymphocytes, which are considered as the major effector cells for protection against the parasite.1,3,4

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of Toxoplasma transferrins-binding proteins since the acquisition of iron mainly from host iron-transporting proteins (transferrin or lactoferrin) is considered as one of the most important mechanisms essential for parasites growth during infection. The performed study revealed the members of ROP2-rhoptry protein family, ROP2 and ROP4 proteins (Fig. 2), as ligands for human lactoferrin indicating these antigens as potentially involved in the uptake of iron from the infected host. This fact, together with the findings of other laboratories showing the expression of ROP2 and ROP4 in three life-stages of T. gondii (tachyzoites, bradyzoites and sporozoites), their contribution to many fundamental functions for Toxoplasma host cell invasion (e.g., PV formation, modulation of PV membrane properties, interaction with the mitochondrial import machinery, etc.) and their documented immunogenic potential, made these antigens very valuable candidates for further studies. To overcome disadvantages associated with the administration of live attenuated parasites, such as morbidity and mortality in vaccinees, we decided to use a subunit vaccine composed of recombinant forms of Toxoplasma proteins. Moreover, since bivalent vaccine comprising recombinant forms of ROP2 and ROP4 showed rather a low level of protection (46% of reduction of the brain cysts number), we enriched the cocktail of the rhoptry antigens with recombinant SAG1 and GRA4 proteins (Fig. 2). The latter proteins play an essential role in T. gondii immunogenicity and pathogenicity. The SAG1 protein is a well-defined, highly immunogenic main surface antigen of tachyzoites considered as an essential element of the parasite attachment to the host cell. Dense granule protein GRA4 is a secretory protein characteristic for Toxoplasma tachyzoites, bradyzoites and sporozoites responsible for induction of the protective immunity and modification of the microenvironment inside the PV, which is crucial for intracellularly replicating tachyzoites. The immunogenic and protective efficacy of the three designed vaccines composed of rROP2 + rGRA4 + rSAG1, rROP2 + rROP2 + rGRA4 and rROP2 + rROP2 + rSAG1 was tested in the experimental mouse model of chronic toxoplasmosis in three inbred mouse strains C3/BL/6 (haptotyp H-2^c), C3/HeJ (haptotyp H-2^e) and BALB/c (haptotyp H-2^a), whose different genetic background determines their high, intermediate and low susceptibility to T. gondii infection, respectively. This complex study revealed the protective and immunogenic potential of the studied vaccines dependent on the antigen composition and the genetic background of immunized mice. The highest protection level detected as a decrease in brain cyst formation was induced by the mixture of the rhoptry proteins with rSAG1 antigen and reached 90%, 71% and 77% in C3/BL/6, C3/HeJ and BALB/c mice, respectively. This strong, but still partial, protection was correlated with the generation of vaccine-induced immune response manifested as a synthesis of the systemic antigens-specific IgG antibodies in the sera of vaccinated laboratory animals and increased in vitro production of Th1-type cytokines, IFN-γ and IL-2, by specific antigen-stimulated spleen cells of the immunized mice. In our studies we used the intraperitoneal challenge of mice with the cyst-forming DX strain of T. gondii. Obviously, intraperitoneal administration of the parasite is not natural compared with the oral route of infection, however, it was indicated that such an experimental protocol provides the most reliable and quantitative challenge and has been frequently used in many studies. Although the oral route is the main avenue of T. gondii invasion, it should also be mentioned that the parasite invasions arise in all transplant recipient groups, but at varying frequencies depending on a type of transplanted organs. It was reported that 57% heart, 20% liver, around 1% kidney and 0.5–2% bone marrow transplant recipients who were Toxoplasma-negative before transplantation and who received organs from Toxoplasma-positive donors acquired the primary parasite infection resulting in relatively high morbidity and mortality. Clinically the situation can be more complicated for these patients since the immunosuppressive treatment exposes them to an increased risk of severe life-threatening toxoplasmosis. Additionally, the diagnosis of the parasite invasion in organ transplant recipients is not always unequivocal due to the atypical immune responses. Moreover, in some cases the incidence of T. gondii infection was not even considered and the parasitosis was documented postmortem.

**Future Prospects**

There are several possible strategies used to enhance the effectiveness of the vaccination against toxoplasmosis. The most frequently studied are: (1) inclusion of additional antigens into vaccine formula, (2) usage of new adjuvants directing the immune response of an immunized individual toward Th1-type or (3) mucosal delivery of the subunit vaccine that is more convenient due to the oral route of Toxoplasma infection and stimulation of not only systemic but also local immune response related to the mucosal surfaces. Among different antigens considered as possible tools to improve the protective effect of ROP2 + ROP4 + GRA4/SAG1 vaccination, Toxoplasma microneme and rhoptry neck proteins (RON) seem to be very interesting candidates. Micronemal apical membrane antigen 1 (AMA1) and

**Figure 2.** Toxoplasma gondii tachyzoite with secretory organelles and nucleus.
RON proteins cooperate in the formation of the moving junction, a key tight connection between the parasite and the plasma membrane of the host cell. This structure is crucial for Toxoplasma invasion and influences the PV generation as well as the biochemical properties of the PVM.36,38 Additionally, AMA1 specific antibodies and the reduced expression of AMA1 antigen, due to their protective role in the moving junction development and invasion by T. gondii.18,31 AMA1 protein contribute to a loss of or reduced expression of antibodies and the reduced expression of the increased release of IFN-γ. The increased expression of specific IgG2a or IgG2c antibodies, respectively, and resulted in enhanced cellular immune response associated with the increased release of IFN-γ. Moreover, partial but significant protection against acute toxoplasmosis was noted as a result of ama1 DNA vaccination. Apart from AMA1 antigen, many experimental trials of single- or multi-antigenic/cytokine vaccines against toxoplasmosis revealed transmembrane (MIC2,39,40 MIC6,41 and MIC842) and soluble (MIC1,42 MIC343 and MIC444) microneme proteins as potential stimulators of specific humoral and cellular immune responses able to protect vaccinated mice against acute and chronic Toxoplasma infection. These proteins are also particularly interesting since they are responsible for the parasite gliding motility, adherence to the host cell and serve as essential determinants of the parasite virulence and invasion process (e.g., MIC1,40 MIC2,41 MIC343 and MIC842). In contrast to frequently studied microneme proteins, only one rhoptry neck protein, RON4, was tested in terms of its suitability as a component of a future vaccine against toxoplasmosis. Immunization of C57BL/6 mice with recombinant RON4 proteins (full-length RON4, N-terminal part of RON4 and N-terminal part of RON4) or rron4 gene showed for the first time the immunogenic properties of this Toxoplasma protein related to the development of antigen-specific IgG antibodies and the production of Th1-type cytokines. However, the RON4 or rron4 DNA vaccine failed to successfully protect immunized mice against chronic Toxoplasma infection provided by an oral challenge with 7 cysts of the parasite 76K strain.45 In the future studies the other members of rhoptry neck protein family, RON2, RON5 and RON8, that form a moving junction complex with the micronemal AMA1 antigen, could be also valuable as vaccine antigens. Similarly to micronemal proteins, RON antigens seem to be worth special attention in designing a new improved rROP2 + rROP4 + rGRA4 vaccine due to their protective potential of the subunit vaccines are CpG-oligodeoxynucleotides. Similarly to MPL, they belong to the family of TLR agonists and are evaluated in clinical trials as adjuvants of hepatitis B, hepatitis C, malaria and cancer vaccines.46 Additionally, CpG-oligodeoxynucleotides have been successfully used as an adjuvant of an experimental vaccine against toxoplasmosis. The co-administration of T. gondii recombinant proteins ROP2 and GRA4 with this candidate adjuvant resulted in strong predominant Th1 response manifested as high IgG2a/IgG1 antibody ratio and increased synthesis of IFN-γ.47 The next promising adjuvants able to expand the vaccine-induced-cellular mechanisms are cytokines such as IL-12 and IL-18. Both cytokines skew the immune response toward Th1 pattern and have been shown to be very efficient as components of the experimental anti-Toxoplasma subunit or DNA vaccines.48,49 The strategy of antigen delivery is also an important factor determining the effectiveness of vaccination. Most tested experimental vaccines against toxoplasmosis have been administered intradermally, subcutaneously or intramuscularly depending on the type of the vaccine, the subunit or DNA. Such immunization protocols usually led to the development of systemic immune mechanisms but did not elicit the synthesis of secretory IgA antibodies, which are produced by the cells of mucosal tissues and are responsible for defense within the mucosal surfaces. Stimulation of mucosal immune response seems to be the favorable in the case of vaccines against pathogens interacting with host mucosae. Furthermore, the presence of immunocompetent repressing B and T lymphocytes in the lamina propria of the epithelia creates a possibility of inducing an antigen-specific immune response in areas distant from the site of antigen
The natural oral route of T. gondii infection suggests that mucosal administration of antigens is more adequate compared with subcutaneous immunization. Additionally, by promoting both local and systemic immune responses, mucosal vaccination could improve the immunogenic and protective potential of the studied vaccines. Intranasal immunization of mice with immunoaffinity-purified Toxoplasma SAG1 antigen, proved that mucosal vaccination could improve the immunogenic and protective potential of the studied vaccines. Intranasal immunization of mice with immunoaffinity-purified Toxoplasma SAG1 antigen, recombinant rROP2 + rGRA5 + rGRA7 proteins or DNA vaccine comprised of pogo-punc54 showed that mucosal vaccination is a realistic strategy in immunophylaxis of toxoplasmosis. Each tested vaccine provided significant production of antigen-specific IgA and IgG antibodies and Th1-type cytokines. Moreover, the increase in cellular and humoral immune responses was correlated with a partial but substantial level of protection against chronic or acute T. gondii infection.

Another interesting and promising strategy developed in the last decade seems to be implementation of live attenuated microorganisms as vectors for delivery of Toxoplasma antigens. An attractive candidate adapted for this strategy is Mycobacterium bovis Bacille Calmette Guerin (BCG), a vaccine strain providing protection against tuberculosis. This strain has been used for human immunization since 1921 and has a long-established safety profile. Moreover, BCG strain possesses an outstanding adjuvant activity related to its capability to penetrate host cells and replicate intracellularly and to the presence of pathogen-associated molecular patterns (PAMPs) recognized by host pattern recognition receptors (PRRs) engaged in the stimulation of innate cellular immunity. Experiments performed by Supply et al.55 and Wang et al.56 with recombinant M. bovis BCG (BCG) expressing T. gondii GRA1 (BCG/GRA1) or ROP2 (BCG/ROP2) antigen, respectively, revealed the ability of this strategy to induce anti-parasite antigen specific cellular immune response in immunized individuals. However, the immunogenic and protective efficacy of the BCG/GRA1 vaccine was dependent on the immunized hosts. In contrast to BCG/GRA1-vaccinated sheep, the immunized OP1 oocysted mice did not generate specific humoral and cellular immune responses. On the other hand, vaccination of inbred BALB/c mice with BCG/ROP2 provided the partial protection against intraperitoneal challenge with T. gondii RH strain. This protection was correlated with the production of antigen-specific IgG antibodies and the high level of Th1-type cytokines.

In our laboratory we have engineered a recombinant BCG strain expressing Toxoplasma ROP4 protein (unpublished data). The construct was based on an attB integrative vector pMV306 under control of a temperature inducible heat shock protein promoter (P\textsubscript{hsp60}), a starvation inducible promoter (P\textsubscript{fas2}), or an isoniazid inducible fatty acid synthetase promoter (P\textsubscript{pha}). In each construct ROP4 was expressed from the Shine Dalgarno motif of the acetamidase gene was introduced in the front of ROP4. As estimated by western blot analysis with rabbit polyclonal anti-ROP4 antibodies, the highest stable, overproduction of ROP4 was obtained in induced (at 45°C) as well as uninduced cells of rBCG expressing rop4 controlled by the P\textsubscript{hsp60} promoter (Fig. 3). Furthermore, the ROP4 protein was also detectable under inducible conditions, the stationary phase of the temperature induced mycobacterial cells or the presence of isoniazid at a concentration of 2 μg/ml, in rBCG controlled by the P\textsubscript{fas2} or P\textsubscript{pha} promoter, respectively. Preliminary
studies aimed at determining the immuno-
genetic potential of the engineered rBCG strains demonstrated that only M. bovis carrying rpoB gene controlled by the Pcri promoter induced in intraperitoneally vacci-
cinated (2 × 10^7 bacilli/mouse) C57BL/6J mice a significant synthesis of the systemic antigen-specific IgG antibodies compared with their level noted in the sera of control animals immunized with the BCG carry-
ing an empty vector or a parental vaccine BCG strain. The rBCG strains described above are under further analysis in order to determine an exact optimal number of the bacteria used for immunization, an optimal route of delivery and a capabil-
ity to induce cellular as well as humoral immune response in vaccinated laboratory animals.

Conclusions

The construction of an effective vaccine against toxoplasmosis is still an open issue. Despite numerous vaccination tri-
als, only partial protection against acute and chronic T. gondii infection has been obtained. However, in some studies the high level of immunoprotection was noticed. One of the most crucial fac-
tors influencing the efficacy of vaccina-
tion against toxoplasmosis is inclusion of antigens specific for three infectious Toxoplasma life-stages required for the induction of long-lasting cellular and humoral immune responses accompanied by protective immunity, which is neces-
sary for controlling the parasite growth in the infected host and for the successful elimi-
nation of the parasite. Then, further extensive investigations should be performed to develop improved tools for immunoprophylaxis of toxoplasmosis.

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