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Prospects for immunotherapy and vaccines against *Cryptosporidium*

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Cryptosporidium spp is a ubiquitous parasite that has long been recognized as a frequent cause of protozoal diarrhea in humans. While infections in immunocompetent hosts are usually self-limiting, immunocompromised individuals can develop severe, chronic, and life-threatening illness. Vaccine development or immunotherapy that prevents disease or reduces the severity of infection is a relevant option since efficacious drug treatments are lacking. In particular, children in developing countries might benefit the most from a vaccine since cryptosporidiosis in early childhood has been reported to be associated with subsequent impairment in growth, physical fitness, and intellectual capacity. In this review, immunotherapies that have been used clinically are described as well as experimental vaccines and their evaluation in vivo.

Introduction

Cryptosporidium spp. is a protozoan parasite that infects the epithelial cells of the small intestine, causing diarrheal illness in humans. This ubiquitous parasite has long been recognized as one of the most frequent causes of protozoal diarrhea in humans.[1] Outbreaks of cryptosporidial diarrhea in the US and abroad are usually due to contaminated drinking water or food.[2] Most human disease is caused by 1 of 2 species of *Cryptosporidium*: *C. hominis* which is transmitted primarily person to person or *C. parvum*, a species that can be transmitted person to person or zoonotically. In the general population, the cryptosporidial seropositivity rate in humans is high and reported to be anywhere from 25% to >60% depending on the location and population being surveyed.[3,4]

While cryptosporidiosis can be serious in immunocompetent people, it can be devastating to those that are immunocompromised. In AIDS patients, symptoms may include chronic or protracted diarrhea that can become life threatening. Infections among HIV-infected individuals may also become extra-intestinal, spreading to other sites including the gall bladder, biliary tract, pancreas, and pulmonary system.[5] The introduction and widespread use of highly-active anti-retroviral therapy (HAART) in AIDS patients has resulted in a decrease in opportunistic infections, however many adults and children living with HIV/AIDS in sub-Saharan Africa are currently not being treated with ART. Prevalence of *Cryptosporidium* among HIV-positive children with diarrhea has been reported to range between 13% and 74% in sub-Saharan Africa.[6] An early study of the impact of HAART on AIDS-defining illnesses in HIV-infected patients noted a 60% decrease in the incidence of cryptosporidiosis.[7] The development of drug resistance may result in rebounding viral loads and, ultimately, increases in opportunistic infections.

In children in the developing world, malnutrition can significantly lead to higher rates of infection.[8,9] Even a single episode of cryptosporidiosis can result in growth deficits,[10,11] especially during the first 2 y of life, and impact growth long-term.[12] Recently, the Global Enteric Multicenter Study (GEMS) of children under 5-y-old in developing countries found *Cryptosporidium* to be among the top 4 causes of moderate-to-severe diarrhea and that such diarrhea is a "high risk factor for linear growth faltering and death"[13].

Adequate therapies to clear the host of these parasites are lacking despite intensive efforts, including the development of workable experimental models and testing of hundreds of chemotherapeutic agents. Therefore, use of alternative immunotherapies or development of a vaccine that would provide protection or at least reduce severity and longevity of infections would be highly desirable. Among the more important groups in need of a vaccine, as described above, are individuals infected with human immunodeficiency virus (HIV) and children in the developing world.

Immune Responses Elicited by Cryptosporidium Infection

Before an immunotherapy or vaccine is developed, a better understanding of the type of immune responses that induce productive and protective responses are needed. Innate immune responses are important in controlling the infection level of cryptosporidiosis and setting the stage for the adaptive response that follows. Upon infection of the host intestinal epithelial cells, innate receptors respond by generating cytokines and upregulating chemokines that attract and activate other immune cells. Injury to the intestinal epithelial architecture due to infection and inflammation can alter tight junctions between the epithelial cells resulting in increases in the uptake of solutes and microbial antigens. *Cryptosporidium* infections cause both increased permeability of the epithelial barrier[14] and induction of innate inflammatory responses. Upregulation of chemokines,

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histocompatibility complex (MHC) class I and class II molecules, and activation of Toll-like receptor (TLR) molecules have been reported in response to cryptosporidial infection.[15,16] Nitric oxide produced through the induction of nitric oxide synthase (iNOS) of epithelial cells is significantly increased in *C. parvum* infection.[17,18] Additionally, the production of antimicrobial peptides and type 1 interferons occur as a result of infection.[19,20]

IFN-γ–dependent responses in both human infections and animal studies are important in innate and protective immune responses.[21,22] In humans, increased amounts of IFN-γ are generated in response to cryptosporidial specific antigen[23,24] after prior exposure. A likely source of IFN-γ-dependent responses was reported to be NK cells, however, depletion of NK-cells with anti-asialo-GM1 antibody treatment in these mice[25] or stimulation of NK-cells by IL-2[21] did not seem to affect infection. In humans, NK cells may play more of a role as treatment of PBMCs with IL-15 was observed to increase expression of the NK marker, NKG2D, and enhance lysis of *Cryptosporidium*-infected epithelial cells.[26]

Several different mechanisms of resistance mediated by the cytokine have been proposed. In *Cryptosporidium*-infected cells exposed to exogenous IFN-γ, depletion of intracellular iron was identiðed as a possible mechanism of action responsible for inhibition of *C. parvum* growth.[27] The activation of TNF-α expression via upregulation of its transcription factor NF-κβ by IFN-γ has been suggested as another potential mechanism.[28]

As important as innate immunity is in the initial stages of infection, adaptive immunity is needed to clear the parasites completely. This is evident clinically in that immunocompromised individuals have more severe and potentially-life threatening disease and experimentally as infections in nude and severe combined immunodeficiency (SCID) mice are chronic.[29,30] In particular, $CD4^+$ lymphocytes are crucial for the resolution of infection; patients with $CD4^+$ counts greater than 200 cells/mm^3 tend to have less severe disease than those with less than 50 cells/mm^3[3,31] and mice depleted with anti-$CD4^+$ antibody have markedly decreased immunity antibody.[32] $CD8^+$ T-cells also play an important role in response to infection. The mechanism by which they effect immune responses is not entirely clear but $CD8^+$ T-cells probably contribute to production of cytokines as these cells secrete IFN-γ early in infection[33] and increase their production of IFN-γ when stimulated with the cryptosporidial-specific antigen, gp15.[24,33] Additionally, they may act through cytotoxicity as antigen sensitized $CD8^+$ T cells and reduce the parasite load in infected intestinal epithelial cell cultures by potentially lysing infected intestinal epithelial cells.[34]

Because cryptosporidial infections activate a Th1 inflammatory response, cytokines, such as TNF-α, IFN-γ, IL-18, and IL-12, play an important role in resistance and recovery to infection. In particular, 2 cytokines that promote IFN-γ production are IL-12 and IL-18. In one study, treatment of both immunocompetent and immunodeficient mice with IL-12 before infection prevented or greatly reduced the severity of infection and was attributed to a decrease in IFN-γ reduction.[35] IL-18 is produced by epithelial cells in the gut and a number of different immune cells and is upregulated in response to *C. parvum* infection in vitro[36] and in mice.[37,38] Treatment with rIL-18 also decreases infection,[39] while treatment with anti-IL-18 increases parasite load.[38]

The role of humoral responses against cryptosporidiosis is less clear. Antibody responses, speciðcally IgG and IgA, are mounted against the parasite following primary infections.[40,41] In humans, antibody response is generated in response to infection and correlates with less symptoms.[42] However, in the absence of a cell-mediated immunity humoral responses alone may not be sufficient as AIDS patients with chronic cryptosporidiosis have high titers of IgA. Additionally, mice that lack B cells or have been depleted of B cells are able to recover from infection. The overall clinical and experimental data suggests that antibody plays a role in protection of the host by preventing attachment or by neutralizing parasite molecules involved in invasion, but may not be an essential component for recovery.

Secondary experimental infections with *C. parvum* in animals result in decreased oocyst shedding and reduced parasite colonization compared with primary infections.[43-45] In mice, immunity was abrogated when T cells or $CD4^+$ cells were depleted from primed cells, while depletion of $CD8^+$ cells could reduce the level of protection, suggesting that both $CD4^+$ and, to a lesser extent, $CD8^+$ cells appeared to be involved in resistance to secondary infection.[21] In particular, intestinal epithelial lymphocyte (IEL) cells are an important memory effector cell as adoptive transfer of both $CD4^+$ and $CD8^+$ IELs in SCID mice reduced parasite load and protected against *Cryptosporidium* infection.[46] In mice, distinct subsets of effector and memory $CD4^+$ T cells develop after infection with *C. parvum*, and mediate protective immunity to re-challenge.[47] In humans, T-cell clones isolated from the blood of patients previously infected with *Cryptosporidium* and then stimulated with cryptosporidial antigen fractions or recombinant peptides ex-vivo are predominantly $CD4^+$ $CD45RO^+$ memory cells.[48] These cells were found to be mainly α/β T cells and produced either IFN-γ alone or in combination with IL-4 or IL-5. In addition, challenge infection leads to an increase in immunoglobulin response in mice[49] and pre-existing antibodies was associated with less oocyst shedding in challenged human volunteers.[50]

It is not known how long immunity persists after resolution of cryptosporidial infection. Experiments in human volunteer studies show that individuals challenged 1 y after experimental infection have reduced infection levels and clinical signs of disease but were not completely resistant to re-infection.[42] It may be that there is a gradual decline of memory T cell responses, like that observed in malaria[51] or that protective memory cell responses are not sufficiently robust to provide complete protection following a single infection.

Immunotherapy

Hyperimmune colostrum

Over the years, there has been a great deal of interest in the potential of immunotherapy for cryptosporidiosis. Passive immunotherapy, through the use of hyperimmune colostrum and/

or monoclonal antibodies directed at multiple cryptosporidial antigens has been pursued as a strategy in humans since the late 1980s and resulted in partial reduction of infection severity. Treatment with immune or hyperimmune bovine colostrum has been associated with both success as well as failure.[52-54] These reports included a child with hypogammaglobulinemia,[55] and patients with either hypogammaglobulinemia[56] or AIDS[56-58] and an observational study in Nigeria where HIV-associated diarrhea was alleviated by a 4-wk treatment regimen with a commercial bovine colostrum product.[59]

Several open label studies using hyperimmune bovine colostrum (HBC) to treat AIDS patients with cryptosporidiosis have also been reported. Patients treated with 48 enteric-coated capsules (40 total grams) per day over a 21-d period showed decreases in mean stool weight and stool frequency, although the parasite load was not evaluated.[60] HBC was also used in a placebo-controlled, double-blind, one-way crossover study in which AIDS patients were treated for 1–2 wk with 20 g/day followed by increasing doses to 80 g/day in some patients. No statistical differences were observed in clinical symptoms but a reduction in stool oocysts was reported.[61] The prophylactic effect of hyperimmune bovine anti-*Cryptosporidium* colostrum immunoglobulin (BACI) was evaluated in healthy adults challenged with *C. parvum*.[62] Subjects receiving BACI or nonfat milk placebo had a 100-fold reduction in oocyst excretion as compared with excretion in the baseline group, however no difference was observed between the BACI and nonfat milk placebo treatment groups. In terms of clinical symptoms, no significant differences were observed in the duration of disease, time to onset of diarrhea, and severity of the disease among groups.

Several studies have evaluated colostrum or monoclonal antibodies (mAb) produced against specific antigens, many of these antigens involved in parasite attachment or invasion of host cells. For example, passive protection against cryptosporidiosis was obtained by treating immunosuppressed mice with immune colostrum generated in cows injected with recombinant pCP15/60 plasmid DNA before and after *C. parvum* infection.[63] Immune bovine colostrum induced by immunization with *C. parvum* recombinant protein rC7, which is the C terminus of the Cp23 protein, provided substantial protection against cryptosporidiosis in neonatal calves.[64]

In a more recent study, cows vaccinated with rCP15/60 produced a significantly greater antibody response compared with controls and this response was strongly associated with the subsequent level of colostral antibody. Calves fed rCP15/60-immune colostrum showed a dose-dependent absorption of antibody, also associated with colostral antibody levels.[65] Induction of the antibody was clearly evident but treatment efficacy was not demonstrated. It should be noted that the use of hyperimmune colostrum not only reduced severity of diarrheal disease in farm animals, such as neonatal calves, but could potentially decrease transmission of *C. parvum* to animals and humans in agricultural settings or in developing countries where families and livestock live in close proximity to one another.

Likewise, treatment with different polyclonal or MAbs resulted in the reduction in oocyst shedding as well as easing of clinical symptoms, although colonization still occurred, but at a considerably reduced level.[66-68] One mAb, designated 3E2, which recognized multiple 46 to –770 kDa sporozoite Ags and a 1300-kDa Ag designated CSL, was able to neutralize sporozoite infectivity in vitro and control murine infection in vivo.[69] The 3E2 mAb combined with other antibodies, including anti-GP25–200 and anti-Cp23 demonstrated significant additive protection over that of the individual MAbs, reducing infection levels by 86–93%. In addition, infection was completely prevented in up to 40% of mice administered 3E2 alone or in combination with 3H2 and 1E10 MAbs.[70]

mAb-based immunotherapy has also been used in other ways. An example includes the use of a human CD40 agonist mAb, CP-870 893 to treat 2 X-linked hyper IgM syndrome patients with biliary cryptosporidiosis.[71] The mAb activated B cells and antigen presenting cells (APCs) in vitro, restoring class switch recombination in XHM B cells and inducing cytokine secretion by monocytes. Although specific antibody responses were lacking, frequent dosing in one subject primed T cells to secrete IFN-γ and suppressed oocyst shedding in stools. Nevertheless, oocyst shedding relapse occurred after discontinuation of therapy.

Another antibody-based immunotherapy involved the generation of an antibody-biocide fusion protein. *Cryptosporidium*-specific antibodies were fused with the antimicrobial peptide LL-37 and administered orally to neonatal mice in a prophylactic model of cryptosporidiosis.[72] Infections in treated mice were reduced by as much as 81% in the mucosal epithelium of the gut. When administered simultaneously with oocyst inocula, several versions of antibody fusion proteins that differed in antigen specificity and in the biocide conjugate inhibited parasite growth in mouse intestinal tissue (up to 82%), although none completely prevented infection.

Despite the variable performance of immune colostrum in clinical trials and other experimental antibody-based therapies, immunotherapy may still be useful in conjunction with conventional drug therapy or as a mechanism to decrease the severity of infection in neonatal animals or moderately immunocompromised individuals.

Vaccines

Considerations for generating an effective vaccine

Despite intensive efforts to develop workable experimental models and the evaluation of nearly 1000 chemotherapeutic agents, efficacious therapies that clear the host of these parasites are still lacking. Nitazoxanide (NTZ), a thiazolide drug with reported broad antiparasitic activities, is currently the only FDA-approved drug for use against cryptosporidiosis in immunocompetent patients but is considered ineffective in immunocompromised individuals.[73]

Because of the lack of efficacious drug treatments, vaccine development that prevents disease or reduces the severity of infection is a relevant option. This is particularly true for certain groups such as immunocompromised individuals and children in developing countries since cryptosporidiosis in early childhood

has been reported to be associated with subsequent impairment in growth, physical fitness, and intellectual capacity.[74] Targeting the latter group may also bring several challenges as vaccines may have lower efficacy due to the young age of the child, possible interference by maternal antibodies, micronutrient deficiencies, and persistent exposure to other pathogens.

Since this parasite is localized to the intestinal tract, a vaccine that stimulates mucosal immune responses will likely be necessary. The few commercial mucosal vaccines that exist are typically "live" and attenuated by serial passage, chemical mutagenesis, deletion of virulence genes, or reassortant as is the case of the RotaTeq rotavirus vaccine.[75] Live vaccines have been used successfully against a related parasite, *Eimeria*, which infects intestinal epithelial cells of chickens. These vaccines were attenuated by passage through chick embryos or by screening during development in chickens for genetically stable parasites that have truncated asexual reproduction.[76] While a live, attenuated, vaccine might be feasible for immunocompetent individuals, there may be insurmountable complications for immunocompromised individuals. In particular, the large multiplication of parasite stages associated with asexual stage recycling (and autoinfection by thin-walled oocysts) is compounded by the potentially large number thick-walled oocysts that might be shed, representing a risk of reinfection of the vaccinated individual and infection of other exposed individuals.

Alternatively, it may be possible to design a vaccine that is composed of a few select immunodominant proteins. Because the mucosal immune system in the intestinal tract typically exists in a state of active tolerance to food antigens and commensal bacteria it may be more difficult to achieve a strong immune response with a subunit or non-live vaccine directed at gut pathogens. Strategies to overcome this may need to be employed such as the use of mucosal vaccine adjuvants (e.g., bacterial toxins, TLR ligands, non-TLR immunostimulants) or delivery systems (e.g., nanoparticles, mucoadhesive polymers) that increase the uptake of the vaccine by antigen-presenting cells, M cells, or that are able to enter antigen-presenting cells by different pathways.

It is not known whether differences between the main 2 *Cryptosporidium* species that infect humans, *C. parvum* and *C. hominis,* will be problematic when developing a vaccine. The homology between the 2 species exceeds 95–97% DNA sequence identity,[77] suggesting high protein conservation. Two studies examined antibody responses in Bangladeshi children to 2 *C. parvum* immunodominant antigens, the Cp23 and Cp15/17, in order to determine differences in immune response to *C. hominis* and *C. parvum*. While most children were infected with *C. hominis*, there were cross-reactive antibody responses to the *C. parvum* antigen, Cp23.[78] Additionally, there was a significant correlation between antibody levels to the immunodominant antigen, Cp15/17, from both from *C. hominis* and *C. parvum*, in spite of polymorphisms in the Cp15/17 sequence. However, in one experimental study, gnotobiotic pigs were first infected with *C. hominis* and then challenge with either *C. parvum* or *C. hominis.* The *C. hominis*–specific immunity was sufficient to completely protect against challenge with the same species while some low level infection was observed with *C. parvum,* suggesting that protection was substantial but not 100%.[79]

Lastly, what minimum level of vaccine efficacy would be acceptable (provide "sufficient" or "adequate" protection), if sterile immunity were not achievable? For example, immunization with rotavirus vaccine achieves approximately 80–90% protective efficacy in developed countries such as the United States and Finland. Although complete protection was not achieved, immunization has resulted in an approximately 50% decrease in hospitalizations for diarrhea in the United States.[80] In developing nations such as in Africa (Ghana, Kenya and Mali) and Asia (Bangladesh and Vietnam) protection rates are much lower, ranging from 43 to 80% rotavirus vaccination 2012.[81] This may be true of any *Cryptosporidium* vaccine developed.

Experimental Studies

Attenuated vaccines

Attempts to attenuate *Cryptosporidium* have been limited. As stated above, this is in part due to the inability to continuously propagate the parasite in vitro, making genetic manipulation of the parasite (e.g transgenic, mutants) difficult. One method that has been used is γ-irradiation treatment of oocysts or sporozoites.[44,52,82] Attenuation is a challenge since too much radiation kills the parasite, preventing infection of epithelial cells in the intestinal tract, whereas too little radiation would allow complete development of all life cycle stages. In one study, exposure to irradiated oocysts in calves was shown to significantly reduce parasite reproduction while inducing partial resistance to reinfection. Oocysts exposed to 400 Gy were incapable of any measurable development but retained the capacity to elicit a protective response against *C. parvum* challenge.[52] However, protection was only observed in calves re-challenged at 3 wk post infection and not as early as 2 wk post infection, suggesting that immune status at the time of vaccination in neonatal animals may be important in eliciting protective immune responses.

Antigens and potential vaccine candidates

Development of subunit vaccines require the identification of candidate antigen(s). Numerous immunogenic antigens of the *C. parvum* invasive stages involved in attachment or penetration of host cells have been identified (reviewed in 83). Several cryptosporidial antigens are immunodominant; some are surface and/or apical complex proteins that may mediate attachment and invasion. Sera from infected animals and humans recognize a number of immunodominant sporozoite antigens, including polypeptides of approximately 11, 15, 23, 44, 100, 180 and >200.[83] These include the surface antigens CSL, Cp900, Cp23/27, Cp40/45, Cp15/17, Muc4 and Muc5, some of which are partially or heavily glycosylated. Antibodies developed against some of these antigens demonstrated therapeutic efficacy in mouse and animal models. Much of this work has focused on the Cp15 and Cp23 antigens. The Cp40/15, is expressed as a precursor glycoprotein (CP60) that is proteolytically processed[84] to yield mature glycopeptides Cp40/15 and Cp15/17, which remain noncovalently associated following cleavage.[85] The C-terminal Cp15/17 peptide

is anchored to the membrane via a glycosylphosphatidylinositol linkage,[86]localized to the surface of zoites, and is shed in trails during gliding motility[87]. Cp15/17 is an immunodominant protein consistently recognized by sera from infected persons. Cp23/27 is a surface protein expressed on the invasive stages of the parasite, is shed in trails during gliding motility.[88,89] Like Cp15/17, Cp23 is an immunodominant protein and antibodies to it are frequently detected following *Cryptosporidium* infection[3] In a study of experimentally-infected human volunteers, those that had pre-existing serum IgG to the Cp23/27-kDa antigen excreted fewer oocysts compared with those that did not.

Identification of additional antigens could aid vaccine development by a including efficacious targets or by incorporating multiple antigens or antigenic epitopes. In particular, little is known about the sexual stages of *Cryptosporidium* species. Reverse vaccinology uses high-throughput in silico screening of the entire genome of a pathogen to identify genes that encode proteins with the attributes of good vaccine targets. This offers an approach that may be useful, particularly for organisms like *Cryptosporidium* that are not easy to culture. The discovery of an important malaria stage-specific gene *UIS3*, was accomplished using gene-profiling studies and subsequently developed into the GAS vaccine.[90] One of the drawbacks to this approach is that while the genomes of both *C. parvum* and *C. hominis* have been sequenced, the genomes have not been fully annotated and many hypothetical proteins have been identified where no experimentally-expressed evidence exists.[91] More data on protein expression and relative protein expression in the different life cycle stages would be helpful, in addition to genomic information and bioinformatic tools, to establish criteria guiding the identification of appropriate vaccine candidates. Additionally, these targets still need to be prioritized, expressed as recombinant proteins, and tested in appropriate in vitro or in vivo models to assess immunogenicity and protection in in vitro or animal models.

DNA Vaccines

DNA immunization has been used to induce antigen-specific B and T cell responses in various infection model systems.[92-95] The first DNA vaccine expressing the Cp15/60 gene, a sporozoite surface antigen,[96] was injected into the mammary gland of cows. Sera and colostrum that were generated conferred a protective response when evaluated in *Cryptosporidium*-infected cell culture assays and in immunosuppressed mice. It induced primarily a type-1 immune response when injected either intranasally or intramuscularly into mice.[95,97] Intranasal immunization with CP15-DNA induced specific and long lasting production of anti-CP15 IgA in intestinal secretions and specific IgG in the sera of mice which persisted for up to 1 y after the first DNA inoculation.[95] Efficacy has also been demonstrated by the generation of Cp23-specific immune responses: mice immunized with Cp23-DNA developed partial protection against *C. parvum* infection as shown by the >60% reduction in oocyst shedding after challenge.[98] In another study, administration of a DNA vaccine encoding *C. parvum* Cp15 and Cp23 resulted in induction of Th1 immune responses and increased resistance to infection.[99]

Evaluation of a DNA vaccine comprised of P2 (CpP2), which may be an important marker of repeated exposure to *C. parvum* infection,[100] showed that CpP2-DNA followed by immunization with P2 protein (prime-boost), significantly increase antibody production compared with immunization with just the protein or CpP2-DNA alone. When challenged, reduction in oocysts production was not statistically significant, although a trend in reduced infection was observed in the CpP2-DNA-immunized mice.[101]

Using Attenuated Bacteria Vectors

Attenuated *Salmonella* vaccines offer a number of advantages including the fact that they induce both cell-mediated and humoral responses, elicit a systemic and local response, are easy to administer, and are affordable.[102] Depending on the strain, *Salmonella* vectors can also have a broad host range that can be used for both human and veterinary uses. They have been used successfully to deliver heterologous antigens for a number of organisms including intestinal parasite species such as *Toxoplasma gondii* and *Eimeria tenella*.[103,104]

The attenuated *Salmonella enterica* serovar Typhimurium vaccine strain SL3261 was first used as an antigen delivery system for the oral immunization of mice against 2 *Cryptosporidium parvum* antigens, Cp23 and Cp40.[105] Each antigen was subcloned into the pTECH1 vector system, which allows them to be expressed as fusion proteins with the highly immunogenic fragment C of tetanus toxin under the control of the anaerobically inducible *nirB* promoter. Specific serum immunoglobulin G (IgG) antibodies against the Cp23 or Cp40 antigen were detected by enzyme-linked immunosorbent assay 35 d after immunization. Also, serum IgA and mucosal (feces) IgA antibodies were detected in 30% of the mice immunized with Cp23. In addition, prime-boosting with Cp23 and Cp40 DNA vaccine vectors followed by *Salmonella* immunization significantly increased antibody responses to both antigens.

In another study, 3 antigens, Cp15, profilin, and a *Cryptosporidium* apyrase, were delivered in a heterologous prime-boost regimen as fusions with cytolysin A (ClyA) in a *Salmonella* live vaccine vector and as purified recombinant antigens, and were found to induce specific and potent humoral and cellular immune responses.[106] Profilin is a potent inducer of immune responses in mice by both *Eimeria* and *Toxoplasma* parasites and works through the toll receptor TRL11. An analogous receptor (TRL11) has not been found in humans, so it is unclear if responses would be similar in humans. In another study, a prime-boost immunization regimen using an intranasal route followed by oral *Salmonella* live vaccine vector of the Cp15 antigen increased immune responses but did not result in decreased infection.[107]

Using Other Vectors for Expression or as a Vaccine Vector

T. gondii has the ability to function as an expression system and antigen delivery system of heterologous proteins for related apicomplexan pathogens. In primate malaria, tachyzoites expressing the *Plasmodium knowlesi* circumsporozoite (CS) protein were able to elicit a response in primates after immunization.[108] In rodent malaria, *T. gondii* was used as a vaccine vehicle for priming $CD8^+$-dependent cell-mediated immunity against challenge with *Plasmodium yoelii*.[108,109] It was anticipated that because *C. parvum* and *T. gondii* are closely related apicomplexans, that *C. parvum* antigens expressed in *T. gondii* most likely have a similar structural conformation, especially with antigens that have significant glycosylation.[110] Cp23 was stably expressed in *T. gondii* (Tg/P23) and its protective effects were evaluated in a mouse model. Mice inoculated with lysed Tg/P23 induced a specific anti-P23 response with the production of a high level of serum IgG, and its subclass responses were IgG1 dominant.[111] While *T. gondii* may be useful for generating recombinant antigens, the use of live, attenuated *T. gondii* vaccines in humans is difficult to envisage due to side effects and risks for breakthrough infection.[112]

It is possible that other vectors (e.g., *Listeria*, adenovirus) may increase vaccine efficacy or have additional beneficial effects such as the use of a probiotic. For example, *Lactobacillus casei* was used to stably express the Cp23 and was evaluated for immunogenicity in a mouse model.[113] Additionally, the use of genetically engineered yeast vectors, including *S. boulardii* has been proposed.[114] It has the advantage over other yeast (e.g., *S. cerevisiae*) of greater resistance to acidic conditions (e.g., in the stomach) and higher temperatures. This probiotic can be genetically modified to contain constructs which express the vaccine candidate in conjunction with an adjuvant and Fc portion of an antibody molecule so that it can be more efficiently taken up by antigen presenting cells in the Peyer's patches in the intestinal tract and generate a better mucosal immune response (T. Lamb, personal communications).

Lastly, the use of adjuvants might improve the response of less than ideal vaccines. Some adjuvants rely on TLR ligands, like oligodeoxynucleotides (ODNs) that have the potential of stimulating the immune system through toll receptors[115] or genes for cytokines (e.g., IL-12 or GM-CSF) that when expressed would boost immune signals. In one study, intraperitoneal and oral pretreatment with one oligodeoxynucleotide, CpG ODN 1668 led to a strong initial upregulation of cytokines and CD69 mRNA in the intestine and a decrease in parasite load by a Toll-like receptor 9 (TLR9)-dependent mechanism.[116] Additionally, use of cytokines, such as IL-18 and IL-12, may increase response by inducing a TH1 response. As an example, co-immunization with the multivalent DNA and pMEM12R plasmid encoding IL-12 was able to further enhance these responses compared with a multivalent DNA *Cryptosporidium* vaccine alone.[99]

Future Directions for Cryptosporidium Research

Understanding host-parasite interactions and the essential elements of immunity to *Cryptosporidium* spp. may lead to the development of effective immunotherapies or vaccines. The continuing increase in genome sequence data should aid in the identification and characterization of antigens and potential vaccine candidates. Of particular value will be increasing our knowledge of the expression of proteins during the different life cycle stages. Identification of other vaccine targets, multi-antigen formulations or constructs, or use of an attenuated *Cryptosporidium* strain could result in better immunological responses and protection from symptomatic disease and/or infection. The ability to generate knockouts/attenuated parasites and identification of effective cryopreservation methods would aid in the development of potential vaccine strains. Active research in this area will hopefully overcome some of the barriers to success and more efficacious therapies and vaccines will be developed to treat this potentially severe disease

Disclosure of Potential Conflicts of Interests

No potential conflicts of interest were disclosed.

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