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Speculation on whether a vaccine against cryptosporidiosis is a reality or fantasy

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Abstract

In this paper the authors question whether the development of a vaccine against cryptosporidiosis could be taken into consideration. The necessity and feasibility of such a vaccine for human and veterinary application is discussed. Developmental stages within the life cycle of the parasite that might act as possible targets for vaccine development are summarised, as well as the target antigens offered by molecular biology and immunology studies. Vaccination trials against cryptosporidiosis carried out so far, including the active and passive immunisation approach, are also overviewed. It seems that with respect to a *Cryptosporidium* vaccine two target groups can be considered: children of the developing world and neonatal ruminants. Antigens representing possible candidates for a subunit vaccine were identified based on their function, location and/or the immune response they evoke. While the active vaccination of newborn calves, lambs and goat kids has to face a number of important limitations, the passive immunisation approach, where dams were immunised to protect their progeny by colostral transfer, was proven to be a valuable alternative. Finally, a number of points of action for the near future are put forward. © 1999 Australian Society for Parasitology Inc. Published by Elsevier Science Ltd. All rights reserved.

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1. Introduction

In the past two decades, the increasing recognition of cryptosporidiosis as a harmful, difficult to control, infectious disease of humans and animals, has been paralleled by the number of scientific papers on this subject. Given the emergence of the medical and economic impact of this disease, and the rise of knowledge on the biology of *Cryptosporidium* spp. and their interactions with the host, one might question whether the development of a vaccine against cryptosporidiosis

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should be pursued. Here, five members of the Working Group III (Cryptosporidiosis and cyclosporosis) of the COST-820 Action of the Commission of the European Union, will give an overview of the scientific knowledge that is relevant with respect to this question. The commercial aspects of a *Cryptosporidium* vaccine will not be mentioned, as we realise that the successful commercialisation of a novel research finding by converting it into a licensed vaccine, will confront obstacles in addition to the scientific work [1].

2. A vaccine for human cryptosporidiosis

The first question that should be asked before considering the development of a vaccine for human cryptosporidiosis is: "is such a vaccine necessary?"; in other words, do the frequency and severity of human cryptosporidiosis justify the development of a vaccine, and which populations should benefit from a vaccinal strategy? The second question is whether acquisition of vaccinal protection against *Cryptosporidium parvum* is feasible.

2.1. Necessity

Answering the first question needs to take into account the frequency and severity of human cryptosporidiosis. Seroepidemiological studies indicate that transmission of *C. parvum* to human populations is frequent [2], especially in the developing world, with anti-*Cryptosporidium* antibodies being detected in the sera of 15 to 44% of the population in industrialised countries, and in more than 50% of the population in developing countries. In terms of clinical symptoms, an analysis of data from 78 studies showed that altogether, *C. parvum* is detected in faeces in 2.1% of diarrhoeas in industrialised countries and 6.1% in developing countries. In AIDS, a review of 22 studies showed that *Cryptosporidium* infection was diagnosed in 24% of diarrhoeas in developing countries and 13.8% in developed countries [3]. Although the incidence of AIDS cryptosporidiosis has decreased dramatically since the use of triple-antiretroviral therapy ([4], Costagliola D. Trends in incidence of clinical manifestations of HIV infection and antiretroviral prescriptions in French university hospitals. In: 5th Conference on retroviruses and opportunistic infections. Chicago, 1998; Abstract no. 182), *C. parvum* remains a threat to HIV infected patients.

Clinical studies show that *C. parvum* infection may result in severe symptoms. This is a classical feature of cryptosporidiosis in AIDS patients who may experience a chronic drug-resistant cholera-like illness and dissemination of the parasite to the biliary tract or the bronchial tree [5]. In addition, it has also been shown that in young children, *Cryptosporidium* infection is associated with increased diarrhoea, morbidity and malnutrition, and may result in a significant decline in growth [6–8]. Given the importance of diarrhoea on child morbidity and mortality in developing countries, these complications are likely to have a major impact on public health on a world-wide basis.

2.2. Feasibility

The second question that should be asked before considering development of a vaccine for human cryptosporidiosis is: "can protection against clinical cryptosporidiosis be achieved through vaccination?" The most convincing evidence that the immune response is involved in the control of human cryptosporidiosis is the fact that *Cryptosporidium* infection is one of the most devastating opportunistic complications of AIDS. Moreover, it was shown that in AIDS patients, the clinical outcome of cryptosporidiosis is related to the degree of immunosuppression, patients with CD4 counts of $180\,mm^{-3}$ or more being more likely to have self limited-infection [9]. There are also reports of patients who cleared *Cryptosporidium* infection following discontinuation of an immunosuppressive treatment [10]. However, the fact that the immune system is involved in the control of an established *C. parvum* infection does not necessarily imply that the immunity acquired after a primary contact with the parasite will be protective against future infections. Two experimental studies addressing

this question have been published. The first one showed that volunteers rechallenged with 500 oocysts 1 year after a primary exposure to *C. parvum* experienced a clinical illness with the same frequency as that after the primary infection. However, the severity of the symptoms and the intensity of infection were lower after the second infection [11]. Moreover, it was recently demonstrated that upon challenge with 500 to 5000 oocysts, the 50% infectious dose is 1880 oocysts in volunteers with pre-existing anti-*C. parvum* antibodies [12], a more than 20-fold increase compared to the 50% infectious dose obtained in seronegative volunteers [13]. Finally, an epidemiological study performed in a region where cryptosporidiosis is highly endemic [14] showed that asymptomatic *C. parvum* infection is possible, suggesting that repeated exposure to the parasite may result in protective immunity.

2.3. Concluding remarks

Based on this information, should the development of a vaccine for cryptosporidiosis be considered as a public health priority in human medicine? The answer to this question differs according to geographic and economic parameters. In industrialised countries, the control of clinical cryptosporidiosis probably relies on the development of new antiviral chemotherapeutic regimens for HIV infected patients and on improved procedures for water purification, more than on vaccination. On the other hand, a vaccinal approach could eventually be considered in countries with poor sanitary conditions where it is almost impossible to control transmission of the parasite. Indeed in these countries, the impact of cryptosporidiosis on child development, and the appalling cost of antiviral chemotherapy for AIDS patients confer to cryptosporidiosis a significant public health importance. In this respect, a putative *Cryptosporidium* vaccine should be part of a multicomponent vaccine targeting *C. parvum* as well as other gastrointestinal pathogens of public health relevance.

However, additional data are still needed before one can accurately answer the question of the need for a human vaccine. New clinico-epidemiological analyses should be performed in several distinct geographic areas to confirm the impact of cryptosporidiosis on the nutritional status and development of young children. The consequences of combined antiviral chemotherapy on AIDS cryptosporidiosis should also be evaluated with more hindsight. Finally, large population genetic and pathogenicity studies are necessary. Indeed, *C. parvum* isolates have been shown to classify in two genotypes, one being human specific and the other being identified in humans and livestock [15–19], and current data suggest that these genotypes have a distinct virulence [19–21]. Moreover, Widmer et al. [22] recently described a higher degree of polymorphism of *C. parvum* isolates, and showed that recombination between the two main genotypes may occur. In any case, and would a vaccine for human cryptosporidiosis appear as a public health priority in future years, and such decision obviously needs additional information, more studies aimed at determining the genetic structure of *C. parvum* and characterising the pathogenicity of the different genotypes would be a preliminary requirement to ensure the choice of the most appropriate target.

3. A vaccine for animal cryptosporidiosis

The same two questions about its necessity and its feasibility should be addressed before considering the development of a *Cryptosporidium* vaccine for veterinary application.

3.1. Necessity

Cryptosporidium can infect a great variety of farm animals, but the parasite is recognised as economically important for only a few of them. In calves, *C. parvum* is considered as the most commonly detected enteropathogenic agent (reviewed in [23]). The infection is most prevalent at the age of 6–15 days (76.7%) [24] and the main presenting signs of the disease, i.e. diarrhoea associated with profuse shedding of infective oocysts [25], are related to this age-class [26]. Multiple suckler beef herds [27], dairy farms with

multiple-cow maternity facilities [28] and fattening units are at higher risk [29]. Of the many drugs tested so far, only a limited number are effective against *Cryptosporidium* and even less are commercially available [23].

Also in lambs and goat kids, *C. parvum* is the most frequent aetiologic agent involved in outbreaks of diarrhoea (65% of the outbreaks in lambs, 40% in goat kids) [30]. In the absence of other enteropathogens, mortality is higher in lambs than in calves [31] and morbidity can reach 100% [25, 32]. In birds cryptosporidiosis most often manifests as respiratory disease caused by *Cryptosporidium baileyi* [33]. Although avian cryptosporidiosis was long considered as an opportunistic disease, it recently became evident that its economic significance should not be underestimated (reviewed in [23]). In piglets, cryptosporidiosis is most prevalent in weaned, 1–2-month-old animals, but the infections are mostly asymptomatic [23]. In rabbits, experimental *Cryptosporidium* infection can cause mortality in suckling animals, but field outbreaks in this age-class are rarely detected [34]. In weaned rabbits, the infection causes only subclinical enteritis (Peeters JE. Recent advances in intestinal pathology of rabbits and further perspectives. In: 4th Congress of the World Rabbit Science Association, Budapest, Hungary, 1988; pp. 293–313), unless immunosuppressive agents are concurrently present [23].

Given this information, the answer to the first question can be affirmative for certain host species. Thus, yes, the economic losses due to cryptosporidiosis in ruminants, especially in small ruminants, can justify the development of a vaccine. In avian species, cryptosporidiosis is clearly an emerging health problem and development of a vaccine could be very valuable, although it does not receive the highest priority. However, we have to realise that the necessity of a vaccine would not exist if preventive hygienic measures would have been explored more profoundly in animal husbandry [23]. A vaccine against cryptosporidiosis in ruminants would complete the gamut of already commercialised vaccines against enteropathogenic agents, including rotavirus, coronavirus, bovine viral diarrhoea virus, *E. coli* and *Salmonella* spp. (reviewed in [35]). Further, the application of a *Cryptosporidium* vaccine would at least be more environmentally friendly and less subject to the development of resistance than those drugs under development for en masse treatment of newborns. Finally, it should be considered that the introduction of an animal vaccine against *C. parvum* might reduce the environmental contamination and consequently the frequency and/or the entity of waterborn cryptosporidiosis outbreaks in humans.

3.2. Feasibility

What about the second question: is the acquisition of a vaccinal protection against *C. parvum* feasible? In contrast to humans, the problems related to cryptosporidiosis in ruminants are restricted to newborns only. An age-related resistance has been observed in calves and lambs. Older animals raised in isolation of *Cryptosporidium* remain susceptible to infection, but the clinical signs are much less severe compared with what is observed in *Cryptosporidium*-infected newborns [26, 36]. After primary exposure with *C. parvum*, ruminants become fully protected against challenge-infection, although the conception 'primary exposure' should be considered with caution in animal cryptosporidiosis. Indeed, once the animals start excreting oocysts they contaminate their own environment and become 'continuously exposed' to the parasite. Consequently, they will shed oocysts until a protective immunity has been acquired. A great range in clinical illness was observed among animals exposed under similar conditions. After receiving a single experimental infection dose of 1.5×10^6 oocysts at 1–3 days of age, calves excreted oocysts for 4–13 days and diarrhoea varied greatly in duration (from 4 to 17 days) and severity from calf to calf [37]. This variability, which is probably due to the different immunocompetence status of individual calves shortly after birth, most likely reflecting distinct genetic make-ups, can hamper the application of a potential vaccine [38].

Calves, lambs and goat kids show some peculiarities around parturition that certainly will

influence the vaccinal approach. Because the placenta of ruminants is syndesmochorial, transplacental passage of immunoglobulin molecules is totally prevented and the neonates are born virtually agammaglobulinaemic. Newborn animals receive large amounts of passive IgG_1 from the colostrum via intestinal absorption during the first 12 to 24 h of life (reviewed in [39]). This passive immunisation of neonatal ruminants via colostral antibodies has been effectively exploited in many vaccination protocols. Most frequently, elevated antibody levels in (hyperimmune) colostrum were achieved by preparturient vaccination of the dams with toxoids, killed bacteria or inactivated viruses (reviewed in [40]). Also, colostral lymphoid cells cross the gastrointestinal barrier and seem to influence the immune response of the newborn [41]. Further, ruminant colostrum contains a variety of soluble immunomodulatory factors (reviewed in [42]) which could be absorbed into the circulation of the suckling infant immediately after birth. Thus, the functional immunity which results from ingestion of colostrum is not mediated by maternal antibodies (predominantly IgG_1) alone, but probably also by a transfer of maternal lymphoid cells and of immunoregulatory cytokines.

Unlike the passive immunisation of ruminants by colostral transfer from vaccinated dams mentioned above, the active vaccination of newborns has to face an important limitation: the active vaccination of young animals that have detectable serum concentrations of maternal antibodies will not successfully stimulate immunity because maternal antibodies will have a blocking effect on vaccinal antigens [43]. This obstacle can be avoided: (i) by administering colostrum free of antibodies directed against the vaccinal antigens, although we admit that this is difficult to perform in the field and probably only of importance in an experimental approach; (ii) by carefully selecting antigens that are not recognised by maternal antibodies. Here we refer to the development of T-cell vaccines against *Theileria* and *Babesia*, where antigens are selected on their potential to stimulate T-cells and not on their recognition by antibodies [44, 45]; (iii) by administering the vaccine in mucosal or other sites, that are less subjected to neutralisation by maternal antibodies in circulation; or (iv) by nucleic acid vaccination. Moreover, any immune response provoked by an active vaccination of neonates must necessarily be a primary response with both a relative long period of latency and a low concentration of antibodies produced. Since animals can be exposed to *Cryptosporidium* from their day of birth, it is questionable whether an active vaccination is capable of mediating any significant immune protection against such an early infection. However, the *Cryptosporidium* vaccine would not be designed to evoke a sterile protection, but rather to reduce production losses caused by clinical illness.

Several effective vaccines based on the inoculation of living parasites have been developed against protozoan infections (reviewed in [46]). Vaccines against *Eimeria* spp. used lines of parasites repeatedly selected for precocious development in chickens [47] or rabbits [48]. There are to our knowledge two reports of such an approach with respect to *Cryptosporidium* spp., though both without success: oocysts obtained after 20 passages of *C. baileyi* in chicken embryos still caused clinical respiratory disease [49] and *C. parvum* oocysts obtained on the first day of patency and fed to calves for 10 successive generations also missed a selection for precocious development [50]. Moreover, one might question whether it is acceptable to apply a live attenuated parasite strain from a zoonotic agent such as *C. parvum*. There are indeed precedents in veterinary medicine, for instance the commercialised live attenuated vaccines against *Toxoplasma gondii* [51] and *Brucella abortis*, although for the latter the potential risk for humans has been demonstrated [52].

4. Targets for vaccine development within the life cycle of *Cryptosporidium* sp.

There are basically three different groups of potential targets within the monoxenic cycle of the parasite: the oocysts, the extracellular stages, and the intracellular stages.

4.1. Oocysts

Oocysts are the transmission forms of the parasite that are produced by sexual reproduction and which are shed into the environment with the faeces. They consist of the resistant oocyst wall which protects the invasive forms, the sporozoites, from external influences. The suitability of oocysts as target for vaccine development is a questionable point. The oocyst wall is apparently a chemically and physically inert structure, thus unlikely to represent a good target for immunological intervention. However, it cannot be excluded that the attack of the oocyst by appropriate immune effector mechanisms could contrast with the onset of the infection by inhibiting excystation or the binding to putative host ligands by unknown oocyst wall components. Furthermore, it has been shown that the oocyst wall shares immunogenic epitopes with other parasite stages [53, 54], suggesting that antibodies raised against these cross-reactive determinants on the oocyst wall may play a protective role later in the life cycle.

4.2. Zoite stages and microgametes

Sporozoites and merozoites are the invasive stages of the parasite. While sporozoites are released from the oocysts during excystation, merozoites are set free by the infected host cell after asexual development of the parasite. Microgametes are the third form of extracellular stages of *Cryptosporidium* which fertilise the macrogamont leading to zygote formation. The extracellular stages are most vulnerable to the conditions within the host intestine and are inactivated by physical and chemical influences. They have to find and infect a host cell very quickly as they have a short life span. Tzipori and Griffiths [55] argue that due to the limited survival time in the gut lumen, chemotherapeutic and immunotherapeutic agents directed against the extracellular forms of *C. parvum* are unlikely to be highly effective. In regard to neutralising antibodies resulting from vaccination, effective concentrations have to be present at all times and at all possible sites of invasion. Indeed, escape of a single parasite to vaccinal immunity is likely to result in completion of the whole life cycle and the development of clinical disease, a problem similar to that of the pre-erythrocytic vaccine for malaria, that gives an 'all or none' protection [56]. Obviously, such need for a 100% effective vaccination is a major difficulty in this immunisation strategy. Nevertheless, in various reports the effect of neutralising immunoglobulins, including mAb or Ig from hyperimmune bovine colostrum and hen egg, has been demonstrated and the antigens have been localised on the surface of zoite stages, especially sporozoites (reviewed in [57, 58]). Moreover, because zoite stages and microgametes occur freely in the intestinal lumen and are not surrounded by the host microvilli membrane, they are more likely to be phagocytosed and digested by Peyer's patch M-cells, and thus, their components are more likely to be presented to T-cells in a MHC class II-restricted way. $CD4^+$ T-cells, that can respond to this MHC class II-restricted antigen presentation, are shown to play a pivotal role in the clearance of the infection, as will be discussed in Section 6.2. in more detail.

4.3. Intracellular stages

Intracellular stages of *C. parvum* include trophozoites, two types of meronts, microgamonts and macrogamonts. They show a unique location that has been described as intracellular but extracytoplasmic [59], rendering them well protected from the gut lumen by two membrane layers including the host microvilli membrane and the parasitophorous vacuole membrane, in addition to the outer and inner parasite pellicle membranes. Intracellular stages are separated from the host cell cytoplasm by an electron dense band and the feeder organelle membrane. The intracellular forms of the parasite may represent the major targets for the cellular host response. Histological studies revealed cellular infiltration of plasma cells, neutrophils [10] and $CD4^+$ and $CD8^+$ T-cells [60] at the site of infection, although the effector mechanism needs to be clarified. Enterocytes have the potential to present antigens from pathogens they harbour intra-

cellularly, in a MHC class I-restricted way, and so evoke $CD8^+$ T-cell-dependent cytotoxicity. However, there is no evidence that this would occur during *C. parvum* infection.

5. Targets for vaccine development offered by the molecular biology

The advent and extremely fast development of molecular biology had a great impact on many fields of biomedical research, including vaccinology. The advantages offered by molecular biology in vaccine development against parasites are manifold and include: (i) the theoretical possibility to have access to the entire antigenic repertoire of an organism through its genes, overcoming the limitations and constraints of biochemical approaches, hampered by the relative abundance and accessibility of any given parasite molecule; (ii) the availability of prokaryotic and eukaryotic expression systems for the production at reasonable costs of large amounts of recombinant antigen devoid of toxic contaminants; (iii) the direct use of genes as DNA vaccines, and (iv) the possibility to engineer the cloned gene to enhance the immunogenicity of the encoded vaccine target. The success of a molecular approach to the development of a vaccine requires an adequate knowledge of the molecular biology of the organism under study. Proper molecular biology research on *C. parvum* started relatively late, in the early '90s. During this decade the number of DNA sequences deposited in the databases rose from about 40, in 1995, to the current 4000. This dramatic increase, reflecting a general growing interest for this apicomplexan parasite, was mainly due to recently started genome projects which are based on partial sequencing of randomly selected genomic DNA (GST and STS projects) or cDNA (EST projects) clones (more information on the world wide web http://mercury.ebi.ac.uk/parasites/cparv.html).

Furthermore, a HAPPY map of the *C. parvum* genome has recently been completed, offering the possibility of a better understanding of the overall organisation of the parasite genome [61]. These approaches represent an effective way to gene discovery in *C. parvum*. However, despite the considerable amount of novel genetic information provided by these random strategies, the number of *C. parvum* genes/proteins that have been so far characterised extensively, and can therefore be evaluated as potential vaccine targets, is very limited (Table 1). These genes, which were cloned in different laboratories adopting various molecular approaches, include: (i) cytoskeletal components; (ii) nuclear proteins; (iii) enzymes; (iv) factors involved in RNA translation; (v) heat shock proteins; (vi) an oocyst wall constituent; as well as (vii) surface and micronemal antigens of sporozoites. Proteins belonging to the latter class seem to represent, at the present state of knowledge, the best candidates for the development of a vaccine against cryptosporidiosis. This statement takes into account: (i) the peculiarity of their aa sequences, as opposed to other cloned *C. parvum* molecules sharing high homology with host counterparts; (ii) their presence in extracellular parasite stages, implying a higher susceptibility to the humoral immune response; and (iii) their putative involvement in key functions for parasite establishment within the host.

5.1. Surface and micronemal proteins of sporozoites

Six *C. parvum* sporozoite proteins have been cloned so far. Five were localised to either the sporozoite surface, CP15/60 [62, 63], CP15 [64] and P23 [65, 66], or to the micronemes, the thrombospondin-related adhesive protein of *Cryptosporidium*-1 (TRAP-C1) [67] and GP900 [68], while the precise location of the sporozoite cysteine-rich protein (SCRP) (Spano et al., unpublished) is presently under study. CP15/60, CP15 and P23 are low mol. wt. polypeptides of 148, 119 and 111 aa, respectively, whose genes were cloned by screening *C. parvum* expression libraries with mAb or polyclonal antibodies raised against oocyst/sporozoite lysates [63, 64, 66, 69]. Despite the lack of a putative leader peptide for extracellular targeting and of transmembrane or GPI-anchoring signals in the aa sequence of CP15/60, CP15 and P23,

Table 1
Most extensively characterised *Cryptosporidium parvum* proteins

Gene name	Definition/activity	Developmental stage	GenBank accession number
COWP	oocyst wall component	macrogamete, oocyst	Z22537
α-tubulin	cytoskeletal component	all	AF013984
β-tubulin	cytoskeletal component	all	Y12615
actin	cytoskeletal component	all	M86241
eIF-4A	translation factor	all	AF001378
EF-1α	elongation factor	all	U69697
EF-2	elongation factor	all	U21667
Hsp70	heat shock protein	all	U69698
Hsp90	heat shock protein	all	AF038559
RNA pol.	RNA polymerase	all	U95995
pHEM2	zinc finger protein	all	U48717
CppA-E1	cation transporter	all	U65981
HemA	hemolysin	ND	U18120
DHFR-TS	enzyme	all	U41366
RNR-R1	enzyme	all	AF043243
PDI	enzyme	all	U48261
Acetyl-CoA s.	enzyme	all	U24082
TRAP-C1	micronemal protein	sporozoite	AF017267
TRAP-C2	adhesive protein	ND	X77586
GP900	micronemal protein	sporozoite, merozoite	AF068065
CP15/60	surface antigen	sporozoite, merozoite	U22892
CP15	surface antigen	sporozoite, oocyst	L34568
SCRP	adhesive protein	sporozoite	AF061328
P23	surface antigen	sporozoite	U34390

ND = not determined

immunofluorescence analyses suggested that these three proteins are located on the membrane of sporozoites, and in the case of CP15/60 and P23 on the membrane of merozoites [62, 65]. Subcellular fractionation shows that P23 can be found in the micronemes of *C. parvum* sporozoites (Petry et al., unpublished). This evidence is corroborated by the observation that CP15 and P23 are shed from the surface of sporozoites during gliding motility [65, 70]. The subcellular localisation of CP15/60, CP15 and P23 would point to these antigens as possible vaccine candidates. CP15/60, CP15 and P23 are the only surface antigens of *C. parvum* sporozoites cloned to date. However, previous electrophoretic analysis of radioiodinated sporozoites showed that the plasmamembrane of this parasite stage displays about 20 different proteins [71]. This antigenic complexity could be exploited in future searches for novel vaccine targets.

Recent findings on host cell invasion by the zoites of the Apicomplexa shed new light on the functional role, and possibly the immunological potential, of parasite proteins located in the micronemes. These apical organelles are known to play a crucial role in the early steps of invasion, when their content is released at the interface between the zoite and the host cell, leading to specific interactions between micronemal proteins and surface receptors of the host [72, 73]. Due to their involvement in a process of pivotal importance for the establishment of the infection and to their transitory display on the parasite surface upon interaction with the host cell, micronemal proteins are being intensely studied as vaccine candidates, especially in *Plasmodium falciparum*. Two *C. parvum* proteins showing distinct molecular architectures, but sharing the localisation in the micronemes, have been cloned so far, GP900 [74] and TRAP-C1 [67]. The first is a highly glycosylated molecule with a polypep-

tidic backbone of approximately 190 kDa, possessing a putative transmembrane region (TMR), two mucin-like domains, distinct cysteine- and threonine-rich motifs, as well as short aa repeats [74]. The protein, localised in the micronemes of sporozoites and merozoites by immunoEM, was also found by immunofluorescence to be exposed on the sporozoite surface and to be shed during gliding motility. Furthermore, antibodies directed against different domains of GP900 were shown to inhibit sporozoite invasion in vitro, claiming for a direct role of the molecule in host–parasite interaction [74]. The second micronemal protein, TRAP-C1, was recently cloned adopting a PCR-based approach for the identification of *C. parvum* DNA sequences encoding the type I repeat of the human protein thrombospondin (TSP) [67]. The TSP type I repeat is an adhesive aa motif which confers to proteins the ability to bind to sulphated sugars. Before TRAP-C1 cloning, molecules possessing the TSP type I repeat had been described, among protozoans, exclusively in apicomplexan parasites of the genera *Plasmodium*, *Toxoplasma* and *Eimeria*. TRAP-C1 is a 687 aa long polypeptide showing: (i) a leader peptide; (ii) six TSP type I repeats; (iii) a TMS; and (iv) a short cytoplasmic domain. These structural features, along with localisation in the micronemes of sporozoites, are shared by the TSP-related proteins TRAP of *Plasmodium* spp. [75], MIC2 of *T. gondii* [76] and Etp100 of *E. tenella* [77]. The reported involvement of these TRAP-C1 homologues in substrate-dependent locomotion of sporozoites [78], as well as in host cell attachment and invasion [79], suggest that TRAP-C1 should be considered a promising vaccine candidate against cryptosporidiosis. Another *C. parvum* molecule deserving consideration from the point of view of vaccine development is the above mentioned SCRP, which is encoded by a single copy gene expressed at very high levels in *C. parvum* sporozoites (Spano et al., unpublished). The recent cloning of a partial cDNA predicts that SCRP is a transmembrane protein possessing one epidermal-growth factor (EGF)-like domain and three copies of the TSP type I repeat (Spano et al., unpublished). Overall, these structural features suggest that this novel member of the TSP family is most likely involved in the interaction with host ligands.

5.2. Oocyst wall constituents

Preliminary studies indicated that the wall of sporulated oocysts contain up to 17 different proteins [80]. Despite the great number of reports describing the reactivity of mAb with oocyst wall constituents, only one *C. parvum* oocyst wall protein is known. The *Cryptosporidium* oocyst wall protein (COWP) was one of the first *C. parvum* proteins identified by molecular techniques, screening a genomic expression library with an antiserum raised against an oocyst homogenate [81]. This abundant protein of 190 kDa is 1622 aa long, has a typical leader peptide and consists of two aa domains, characterised by distinct but related cystine-rich repeats. ImmunoEM studies showed that COWP is accumulated in the wall-forming bodies of mature macrogametes and eventually incorporated in the inner layer of the oocyst wall [82]. The immunological relevance of COWP in natural infections is not well understood; however, the presence of the protein on the inner oocyst wall seems to preclude, or at least discourage, its use as a vaccine target.

6. Targets for vaccine development offered by the immunology

This paragraph is not intended to review the immune response to *Cryptosporidium* infection but to concentrate on those aspects of the host defence system that are relevant to vaccination strategies and on possible targets offered by the study of the immune response acquired against *Cryptosporidium* infection. Recent reviews have dealt with the immunology of cryptosporidiosis and the reader's interest is directed to some of these articles for further reading [57, 83, 84]. In respect to vaccination, two major parts of the host defence system are likely to be involved in the generation of protective immunity: immunoglobulins and T-cell responses.

6.1. Immunoglobulins

The role of immunoglobulins in the elimination of the parasite is controversial. Some evidence supports the involvement of antibodies in the clearance of the infection, such as: (i) the persistency of cryptosporidiosis in congenitally hypogammaglobulinaemic individuals (see in [57]); (ii) the good temporal association between *C. parvum* oocyst excretion and the amount of specific IgA in the faeces of calves [85, 86] and lambs [87]; and (iii) the protective role of neutralising antibodies as demonstrated by their ability to inhibit invasion both in vivo and in vitro (see in [58]). On the other hand, there are observations arguing against the importance of the antibody response: (i) oocyst shedding patterns do not differ between B-cell-depleted neonatal mice [88] or bursectomised chickens [89] and their controls; (ii) challenged immune calves do not show a secondary mucosal antibody response [85]; and (iii) AIDS patients with persistent cryptosporidiosis produce *C. parvum* specific serum and/or mucosal IgG, IgM and IgA [90].

Theoretically, two mechanisms of antibody action can be distinguished. Firstly, antibodies can act luminally by blocking the parasite's interactions with the host cell and by preventing attachment and/or invasion. This kind of action could greatly be facilitated by effector mechanisms (e.g. complement activation/lysis or opsonophagocytosis, binding to Fc receptors/antibody-dependent cell mediated cytotoxicity) and thus potentially attack all extracellular stages, also the non-invasive ones (outlined in [58]). Secondly, Crabb [58] suggested *Cryptosporidium* specific antibodies might act also intracellularly, according to a novel mechanism recently described in rotavirus infections, where secretory IgA inhibited intracellular virus assembly during transcytosis through the infected epithelial cells [91]. In both mechanisms secretory IgA may represent the most promising Ig isotype, because it has the capacity of transcytosis and in the dimeric form with the secretory component, it is reasonably stable in the environment of the intestinal lumen. Favennec et al. [92] have shown that in AIDS patients with cryptosporidiosis, serum and faecal IgA response were mainly IgA1, a subclass that is primarily generated against polypeptide and glycoproteins, whereas the IgA2 subclass, responding to polysaccharide antigens, was underrepresented.

Whereas the quantitative levels of parasite-specific IgG, IgM and IgA have been determined in many studies, the quality of antibodies to neutralisation-sensitive antigens or epitopes has been poorly studied. Traditionally, the selection of antigens for vaccinal purposes was based on their specific and dominant recognition by antibodies from immune animals. A comprehensive overview of the antigens recognised by immune serum/mucosal antibodies, hyperimmune serum/colostrum or mAb is given by Riggs [57]. We will focus only on the few well studied antigens that originate from screening with antibodies from immune animals.

A 15 kDa antigen was recognised by serum/faecal antibodies from *Cryptosporidium*-infected humans, BALB/c mice, rabbits, lambs, calves, piglets and goat kids [93–95]. This antigen comigrated with the sporozoite surface antigen designated CP15/60 (see Section 5.1), defined by the mAb 5C3 [62]. Oral administration of this anti-CP15/60 IgA mAb to suckling mice provided protection against infection [62]. A recent study compared the antigenicity of two different recombinant forms of CP15/60, one produced in a prokaryotic expression system and the other in a eukaryotic one. The most potent immune response was obtained using the eukaryotic form, possibly due to post-translational modifications [96]. Western blot analyses of *Cryptosporidium* proteins using serum/faecal antibodies from *Cryptosporidium*-infected humans, BALB/c mice, rabbits, lambs, calves, piglets, goat kids and horses, all revealed a 23 kDa immunoreactive polypeptide [87, 93–95]. This antigen comigrated with the protein P23, defined by the mAb C6B6 and localised to the surface of sporozoites ([93], and see Section 5.1). IgG1 [66] and IgA [97] mAb reactive with P23 were shown to have significant anti-cryptosporidial activity in mice.

6.2. T-lymphocytes

Undoubtedly, T-lymphocytes are essential for overcoming cryptosporidiosis, as shown by the fact that athymic nude or SCID mice develop chronic infections and that AIDS patients with a severe drop of $CD4^+$ T-cells are most likely to become seriously ill when infected with *C. parvum*. Passive transfer experiments have shown that $CD4^+$ T-cells could abrogate chronic infection in nude or SCID mice [98–101]. Moreover, in immunocompetent mice infected experimentally with *C. muris*, depletion of $CD4^+$ T-cells with specific mAb resulted in increased infection [102]. Protective immune response to cryptosporidial infection is associated with the production of interferon-gamma (IFN-γ) by parasite-specific $CD4^+$ T-cells typically associated with a T helper 1 response [103]. Natural killer cells as a second source of IFN-γ were postulated in SCID mice that show an initial resistance due to the action of an innate defence effector system [104–106]. Despite the increasing knowledge on the immunology of the parasite–host interaction, the effector mechanisms that lead to sterilising immunity are not known. McDonald and Bancroft [105] discussed how IFN-γ may increase the turnover of the parasitised epithelium which would lead to an acceleration of shedding of these cells from the villi. A second mode of action, involving the IFN-γ-induced killing of *Cryptosporidium* has yet to be demonstrated. Even less is known about the involvement of other cells and cytokines in parasite killing.

The strategy of selecting antigens that provoke a T-cell response, might be an interesting alternative to the more common approach to screen for antigens recognised by parasite-specific antibodies. Using crude *C. parvum* oocyst extract a proliferative lymphocyte response [107] and $CD4^+$ T-cell-dependent IFN-γ production [86, 108] have been described. However, this approach could only attribute stimulating potential to separate antigen fractions [109, 110], but failed so far to discover new T-cell antigens. It seems reasonable to believe that among the already known *C. parvum* antigens, some certainly must have T-cell stimulating potential, as was demonstrated for COWP [108].

7. Vaccination trials against cryptosporidiosis

In the previous sections we listed a number of proteins that, because of their function, location or the immune response they evoke, may represent candidates for a subunit vaccine. However, the protective ability of an antigen or any DNA vaccine construct can only be confirmed in a vaccination trial, comparing the acquired protection against challenge infection of immunised and nonimmunised animals. In this section the work done in this domain with respect to *Cryptosporidium* spp. will be overviewed. We will focus only on trials where vaccines were administered parenterally/orally in order to elicit a protective immune response against subsequent *Cryptosporidium* infection. Therapeutic use of antibodies to combat cryptosporidiosis will not be mentioned, with the exception of the passive immunisations of ruminants, where the mothers were immunised to protect their progeny by colostral transfer.

Attempts to immunise against *C. parvum* have been conducted employing oocyst preparations or adopting a DNA-based approach. However, so far vaccination trials remain restricted to laboratory and domestic animals.

7.1. Passive vaccination

In field conditions, administration of colostrum did not protect calves [85, 111] and lambs [112] against naturally acquired infection. However, both calves [113] and lambs [114] fed colostrum from dams that received an i.m. injection of freeze/thawed *C. parvum* oocysts several weeks before parturition, were partially protected against infection. This so-called hyperimmune colostrum contained high titres of specific antibodies. Jenkins et al. [115] reported that ewes immunised by DNA technology with a plasmid encoding the CP15/60 protein, mounted an IgG humoral response in both serum and colostrum. Animals responded to plasmids either after i.m.

injection or intramammary immunisation of the ewes, but only with high doses (0.5–1.0 mg) of plasmid DNA. Sagodira et al. [116] demonstrated that, when given by the nasal route, doses as low as 0.1 mg plasmid DNA encoding the CP15 surface protein, could induce specific humoral and cellular responses in mice. Intestinal IgA were detected for up to 1 year after immunisation, suggesting that this route of vaccination could induce a longterm response. A durable cellular response was also found, as mesenteric lymph node cells were still proliferating in response to *C. parvum* extract more than 1 year after immunisation. However, none of the two latter studies were designed to determine whether the acquired immune response provided protection against *Cryptosporidium* infection of the dams or their progeny.

There are, to our knowledge, two studies that followed the passive vaccination approach against cryptosporidiosis, using a purified recombinant *Cryptosporidium* antigen or its corresponding gene. Perryman et al. [117] immunised late gestation cows with affinity purified rC7, a recombinant *C. parvum* protein containing the 101 C terminal aa of P23. They found that, following challenge infection, none of the immune colostrum-treated calves developed diarrhoea and that the animals shed significantly fewer oocysts. Sagodira et al. [118] showed that vaccination of goats with the gene encoding the CP15 protein also conferred high level of protection against acute cryptosporidiosis in newborn kids. Neonatal kids born to vaccinated goats shed significantly less oocysts than control kids. The mean duration of oocyst excretion was shorter (5.5 days) in kids born to vaccinated goats than in kids born to control goats (9.4 days). Monitoring of the daily mean weight confirmed that kids born to vaccinated dams were not severely affected by the parasite development. Analysis of the immune response of dams following immunisation revealed that nasal vaccination with the CP15-DNA induced specific IgG and IgA in both serum and colostrum. While the proliferative response of spleen cells was different in protected and unprotected kids, the precise mechanism by which this vaccine reduced diarrhoea and oocyst development remained unclear. In addition to the transfer of antibodies and immune cells by colostrum administration, the authors questioned whether DNA immunisation could have sensitised kids in utero to mount cell-mediated and humoral immune responses [118].

7.2. Active vaccination

Harp and Goff [119] developed a vaccine offering partial protection against *C. parvum* infection in calves. In calves receiving an oral preparation of lyophilised killed *C. parvum* oocysts shortly after birth, the mean duration of diarrhoea and of oocyst shedding was shortened. However, when field tested on a large dairy operation with heavy endemic *C. parvum* infection, the vaccine failed to provide protection [120, 121].

Hornok et al. [122] reported attempts to immunise against avian cryptosporidiosis. Chickens were injected twice i.m. with oocysts-derived *C. baileyi* proteins and challenged orally with 8×10^5 *C. baileyi* oocysts. Total oocyst output of immunised chickens was about 60% of that of the nonimmunised controls. No significant differences in serum antibodies were found, suggesting that other immune mechanisms were involved.

8. Conclusions

There seems to be two target groups for which the development of a vaccine against cryptosporidiosis would be justifiable: the children in developing countries and newborn ruminants. In the first instance a vaccinal strategy might greatly contribute to reduction of a public health threat which cannot be controlled by preventive measures. In the case of farm animals the development of a vaccine would be justified by the economic losses due to cryptosporidiosis in livestock husbandry, especially in neonatal ruminant breeding, and the limited availability of effective drugs. The acquisition of vaccinal immunity by active immunisation is presumably more problematic in newborn calves, lambs and kids, than it is in children. Indeed, since *Cryptosporidium* infection manifests itself so early in the life of

these farm animals, it remains questionable whether an active vaccination is capable of mediating any significant immune protection in time. In addition, the blocking effect of circulating maternal antibodies on the vaccinal antigens, impose a very conscientious selection of the antigens to be administered and of the route of vaccination, when choosing for active immunisation. Passive vaccination, where dams are immunised to protect their progeny by colostral transfer, provides an interesting alternative. Its potential with respect to a *Cryptosporidium* vaccine was confirmed by two recent studies [117, 118].

It seems difficult to predict which of the *Cryptosporidium* life cycle stages represents a good target for immunological intervention. In fact, there is a lack of knowledge of the immune effector mechanisms involved, and of the parasite stages that are attacked, during clearance of the infection. Traditionally, immunology has offered a number of possible target antigens for vaccine development, based on their specific and dominant recognition by host antibodies. Protection against cryptosporidiosis has been clearly shown to depend largely on T-cells, however the strategy of selecting antigens based on their ability to elicit a T-cell response has been explored insufficiently so far. In the study of parasite-derived antigens the molecular approach has offered various advantages when compared with the biochemical ones. The number of *C. parvum* sequences deposited in the databases increased dramatically over the past 4 years, mainly due to genome projects. However, only a limited number of *C. parvum* genes have been sufficiently characterised to date, leaving much genetic information largely unexploited.

Answering the question whether a vaccine against cryptosporidiosis is a reality or fantasy, we can conclude that the possibility of developing a vaccine for livestock mammals appears reasonably realistic. There seems to be a need for such a vaccine, and recent data in calves and goats have shown promising results [117, 118]. Moreover, there has been considerable progress in recent years in the development of mucosal adjuvants and the use of attenuated pathogens as vaccine carriers, and a vaccine against cryptosporidiosis would benefit from this progress [123]. The situation may be different in human medicine, and more data are still necessary to determine whether effective protection to *C. parvum* can be achieved through vaccination, and to define the populations likely to benefit from such a strategy. In any case, we should not lose ground, as the research on vaccines against other Apicomplexa, including malaria, has brought us, after more than two decades, only little success [124].

This overview highlighted a number of possible points of action for the near future: (i) a clinico-epidemiological analysis to confirm the impact of cryptosporidiosis on the nutritional status and development of young children; (ii) a study of the consequences of combined antiviral chemotherapy on AIDS cryptosporidiosis; (iii) the study of the immune effector mechanisms involved in the clearance of the infection; (iv) the identification of *Cryptosporidium* T-cell antigens; (v) the understanding of the mechanism involved in the passive immunisation of neonatal ruminants by colostral transfer; (vi) the identification and complete characterisation of new targets offered by the molecular biology; (vii) the confirmation of the protective potential of candidate vaccine antigens in vaccination trials; and last but not least (viii) the study of the genetic structure of *C. parvum* and the characterisation of the pathogenicity of different genotypes to ensure the choice of the most appropriate target.

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