IMMUNITY AND RESISTANCE TO CRYPTOSPORIDIOSIS: THE INTRICATE WAYS OF AN ENIGMATIC PARASITOSIS

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*Genus Cryptosporidium includes around 30 known apicomplexan parasitic species which infect the gastrointestinal tract and rarely the respiratory system of more than 300 vertebrate animals. The immune response against infection by Cryptosporidium spp. includes all strata of innate and adaptive immunity with differences in their significance. The mucosal immunity, expressed predominantly by the “sentinel” role of epitheliocytes, is fundamental to the resistance against an infection (mainly via activation of the TLR4/NF-κB signalling axis). The vast array of epithelial chemokines and cytokines initiate the local inflammatory processes, attract effector cells and may directly suppress the parasite adhesion. The second line of defence includes IFN-γ production by the NK cells in combination with their innate cytotoxicity against the parasite and the infected epitheliocytes. The adaptive immunity against the parasite depends predominantly on cytotoxic CD4+ Th1-lymphocytes, which makes IFN-γ central to the acquired response too. CD8+ cells aid to some extent the activity of Th1-cells but their involvement is not decisive. While Cryptosporidium infection elicits the synthesis of specific serum and mucosal antibodies, the humoral immunity is of minor importance. In immunocompromised hosts, infants and malnourished children, the mild and usually self-limiting infection can become life-threatening or take a chronic course. It is the second leading cause of fatal diarrhoea in children and one of the major opportunistic pathogens in the continually expanding group of patients with immunodeficiencies and systemic chronic diseases. Unravelling the mechanisms of resistance against Cryptosporidium infection is fundamental for the successful prevention of the disease. **Biomed Rev** 2019;30:37-48*

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INTRODUCTION

Genus *Cryptosporidium* includes around 30 known apicomplexan parasitic species which infect more than 300 vertebrate animals. In humans, the disease is transmitted via a faecal-oral mechanism and is associated predominantly with two species *C. hominis* (anthroponotic) and *C. parvum* (zoonotic). Several other members of *Cryptosporidium* spp. (*C. parvum*, *C. meleagridis*, *C. cuniculus*, *C. canis*, *C. felis*, *et cetera*) have also been recorded in humans, but their epidemiological and clinical significance is minor (1). In 2016 a meta-analysis of the Institute for Health Metrics and Evaluation at the University of Washington, Seattle estimated that cryptosporidiosis is the second leading cause of diarrheal infection in children younger than five years worldwide and leads to more than 60 000 fatalities annually (2). Moreover, the condition is underdiagnosed and unrecognised, and its impact on global children's health, growth and development is severely underestimated (1, 2).

Cryptosporidium species complete their entire life cycle in the covering epithelia of the gastrointestinal or respiratory tract of a single vertebrate host. The infection occurs upon the ingestion of viable oocyst from the environment. In the process of excystation, four infective motile sporozoites are released. The apical complex of the sporozoites adheres to the epithelial plasmalemma, and via multiple signalling pathways induces a rearrangement of the host's actin cytoskeleton, and parasite internalisation (1, 3). The resulting reorganisation of the host-parasite interface leads to the formation of a unique structure – a parasitophorous vacuole, a compartment where *Cryptosporidium* sporozoites and all subsequent stages of the life cycle reside. Therefore, the parasite inhabits a place in the cell that is distinctly separated from both the intestinal lumen and the host's cytoplasm. This unique intracellular and yet extracytoplasmic position of the pathogen protects it against the host's immune responses and plausibly contributes to the antimicrobial drug resistance (1, 4, 5).

Since the first studies of cryptosporidiosis in humans in the last decades of the 20th century, the importance of a properly functioning immune system became apparent for the severity and the propagation of the ongoing infection. Subsequently, many researchers have attempted to uncover the intriguing mechanisms of interaction between the parasite and its host's defence systems.

The present review aims to summarise the current evidence from a plethora of immunological *in vitro* and *in vivo* studies about the role of the innate and adaptive

response in the resistance against the infection caused by the *Cryptosporidium* spp.

THE INNATE RESPONSE OF INTESTINAL MUCOSA

As mentioned earlier, all parasitic stages of *Cryptosporidium* spp. are localised in the epithelial lining of the gastrointestinal and (more rarely) pulmonary systems. The apical compartment of the epithelial cell is the only affected structure in the host organism. Consequently, the *Cryptosporidia* are classified as one of the “minimally invasive” pathogens. That is why the innate immune reactions of the epithelial cells are crucial for the host's resistance against this infection (4, 5).

The first contact between the *Cryptosporidium* sporozoites and the epithelial cell induces an intracellular signalling cascade resulting in massive production of “alarming” cytokines (IL-8, TNF- α and IL-1 β) that are triggering cell-mediated immune reactions and phagocytes-dependent inflammation (Fig. 1) (5, 6). In experiments with human gastrointestinal epithelial cell lines infected with *C. parvum*, the production of the powerful neutrophil attractant IL-8 (CXCL-8) is substantially elevated (7). Infected epithelial cells also excrete IL-18 that acts as a potent IFN- γ -inducing factor (8). In neonatal murine models of *C. parvum* infection, the epithelial cells produce a broad range of chemokines of the CXC and CC classes (CCL2, CCL5, CXCL1, CXCL8, CXCL9, CXCL10 *et cetera*), which facilitate the recruitment of various immune cells to the mucosa (4,9,10). CXCL10 is one of the molecules that play an important role by IFN- γ dependent mobilisation of the dendritic cells (DCs) (9, 10).

Parallel with the cytokine and chemokine release, *C. parvum* infection of the epithelial cells induces the expression of intercellular adhesion molecule-1 (ICAM-1). In a model of cryptosporidiosis in human gastrointestinal epithelial cultures, Gong *et al.* detected a steady microRNA-controlled upregulation of ICAM-1 expression accompanied by increased adhesion of human immortalised T-cells to the infected cells (11).

The massive production of proinflammatory cytokines and chemokines can be attributed to the activation of the Toll-like receptors (TLRs), part of the pattern recognition receptors group. Under experimental conditions, TLRs 2 and 4 in *Cryptosporidium* infected epithelial cells lead to the production of a multitude of interconnected chemokines [IL-8 (CXCL8), MCP-1 (CCL2), MIP-2 α (CXCL2)], prostaglandin E2 *et cetera*) involved in the recruitment of immune cells in the intestinal wall (7, 10, 12).

Figure 1. Main mechanisms of innate response to *Cryptosporidium* infection. The contact of the parasite with toll-like receptors (TLR) on the epithelial cells (Epi) plasmalemma triggers a cascade resulting in the massive production of antimicrobial peptides, cytokines and cell adhesion molecules. NK-cells (NK) and macrophages (Ma), recruited to the site of infection, exert their cytotoxic activity predominantly via IFN- γ production. Activated dendritic cells (DC) support the process through continuous secretion of proinflammatory cytokines.

The TLR4/NF- κ B signalling axis is one of the most extensively studied transduction systems following *C. parvum* invasion of the enterocytes. The light-chain nuclear factor kappa (NF- κ B) controls the expression of various genes responsible for cell differentiation, inflammation and immune responses mainly by initialising the synthesis of a variety of pro-inflammatory cytokines - TNF- α , IL-1 β and IL-8 and ICAM-1 (Fig. 1). Activation of the NF- κ B pathway in epithelial cells after the infection with *Cryptosporidium* spp. comprises of early and transient reaction against the

lipopolysaccharides followed by “late” sustained response when the parasite-epithelial interface is completely formed (13–15).

Another major and very interesting role of NF- κ B produced by epithelial cells (and in later stages by activated B-lymphocytes) is its involvement in the apoptosis (5, 7, 13, 14). The NF- κ B triggers the synthesis of a variety of anti-apoptotic factors and postpones the cell death of the infected enterocytes significantly. On the contrary, a severely enhanced Fas-mediated activation of caspase-3 in the adjacent uninfected

cells elicits the pathomorphological changes observed in the intestinal mucosa (7, 13, 14). This protection of the *Cryptosporidium* infected cells from apoptosis is vital for the parasite survival and the propagation of the infection, and one of the primary mechanisms for evasion of host immunity and defence (7, 10, 13, 14).

The enterocytes produce a variety of antimicrobial peptides (e.g. defensins), that can penetrate the cell wall of protozoal and bacterial cells, directly killing the pathogens. *C. parvum* selectively up-regulates human β -defensin1 and -2 peptides (via TLRs mediated signals) leading to the prevention of the *in vitro* development of the *Cryptosporidium* sporozoites or their direct destruction (10, 14).

Thus, epithelial cells play a crucial role in the resistance against cryptosporidiosis by triggering inflammatory responses and mobilisation of leukocytes from the connective tissue and circulation, and cytokine related local parasitotoxic reactions. However, there is a notable difference in the responses of *in vitro* epithelial cell lines as compared to the reactions of the mucosa in the infected animal and human hosts (10). This observation warrants a more in-depth analysis of the signalling pathways involved in the complex interaction between *Cryptosporidia*, epithelial cells and surrounding tissues.

The natural cytotoxicity of the natural killer (NK) cells is just a part of their intricate involvement in the innate reaction against intracellular microorganisms (parasites, bacteria and viruses). NK lymphocytes express a complex array of activating (e.g. IL-2R, IL-12R, IL-18R *et cetera*) and dampening (e.g. inhibitory MHC class I) receptors, which regulate their cytopathic effect and recognise specific ligands produced by the infected or damaged cells. After activation, NK cells exert cytotoxic activity either via the release of directly cytolytic substances (e.g. perforin, granzyme B, FasL *et cetera*), activation of death receptors and the external apoptotic way in target cells or indirectly through the production of various cytokines – predominantly TNF- α , IFN- γ , GM-CSF *et cetera* (16). Experiments have shown that murine lines with a mutation leading to a defect in the natural cytotoxicity of NK cells have a more severe manifestation of *C. parvum* infection as compared to wild type (6). Multiple immunological studies demonstrate that the resistance against *C. parvum* infection is mostly IFN- γ -dependent (4, 7, 17–20). Thus, the NK cells may exhibit their anti-*Cryptosporidium* function via the substantial release of IFN- γ (Fig. 1). This conclusion is supported by evidence of amplified cytotoxicity of the NK cells against *C. parvum*-infected human enterocytes,

especially after incubation with IL-15 (a cytokine responsible for NK maturation and activation) (6, 20).

A subject of substantial interest is how *C. parvum* is presented to the cells of the adaptive immunity if it occupies only the parasitophorous vacuole in the apical part of the enterocytes and does not infect deeper layers of the mucosa. Despite that, studies of biopsy materials from heavily infected AIDS-patients have shown the presence of *C. parvum* in M-cells, DCs, macrophages and other inflammatory cells which confirms their role as antigen-presenting structures (4, 21, 22). The involvement of the DCs in *Cryptosporidium* infection is still under investigation. Several studies demonstrate that when bone marrow DCs are exposed to sporozoites or cryptosporidium antigens, it induces their maturation and starts the production of IFN- α and - β , IL-1 β , IL-6 and IL-12 (10, 20). *C. parvum*-infected murine DCs quickly migrate to the site of infection and, once activated, relocate in the mesenteric lymph nodes (MLNs) (23). Some studies suggest that infected epitheliocytes themselves act as antigen-presenting cell or indirectly facilitate the process via the substantial array of produced cytokines and chemokines that recruit other immune cells (5). Another hypothesis is that the antigen presentation is triggered by the macrophages when they come to phagocytise the apoptotic bystander cells (5, 13). Experiments with neonate mice infected with *C. parvum* have shown a 60-fold rise in the inflammatory monocytes/macrophages, not only in *lamina propria* but also in the sub-epithelium stratum, and the ability of those cells to migrate among the epitheliocytes and sample apoptotic material from the lumen (10). Several recent studies mutually suggest an essential protective mechanism against cryptosporidiosis in which the synergistic initiation of macrophages by IL-12 and IL-18 leads to the production of the vital cytokine IFN- γ (4).

The activation of T-lymphocytes in the intestinal mucosa is a major part of the immune response and structurally takes place in three different locations. The specific reactions against *Cryptosporidium* spp. are probably triggered in the Peyer's patches, where M-cells transport the antigens to the DCs, which phagocytise and subsequently present them to the T-cells. Ultrastructural studies in guinea pigs show that *Cryptosporidia* invade the M-cells of the Peyer's plaques. Parasites are even detected within the phagocytic cells under the mucosa (10, 21). In other experiments with mice infected with *C. parvum* a tenfold increase is observed in the numbers of T- and B- lymphocytes in the Peyer's plaques and parasite's antigens are detected even in remote MLNs (6). A more current

study demonstrates that even living *C. parvum* forms can be detected in the MLNs, verified by the existence of parasitic mRNA (23).

Lamina propria is the second important location of antigen presentation as proven by the serious increase in the lymphocyte populations in the regions overlaid by cryptosporidium invaded enterocytes (24). Furthermore, the recovery of patients with AIDS and chronic diarrhoea on highly active antiretroviral therapy (HAART) coincides with the rapid increase in CD4⁺ cells, specifically within *lamina propria* (25). The importance of the intraepithelial lymphocytes in the control of the cryptosporidiosis is demonstrated by their significant increase recorded in experimental studies in infected mice and cattle (7). Also, severe combined immunodeficiency (SCID) mice inoculated with intraepithelial lymphocytes from healthy donors previously infected with *C. muris* very quickly resolve the *Cryptosporidium* infection (4).

Different studies are trying to estimate the role of other molecules and pathways, such as mucins, exosomes, complement system (C3, C4, and mannose-binding lectin), epithelial cytokines (IL-25, IL-33 and IL-34), serum amyloid A3, osteoprotegerin, thymic stromal lymphopoietin and others, proven to be necessary in the innate mucosal immunity against different microbial pathogens but the results are still preliminary, insufficient and sometimes controversial (4, 5, 7, 10, 26).

THE ROLE OF THE CYTOKINES

The cytokines include numerous effector peptides secreted by a broad array of immunocompetent and other cells. Majority of these molecules are secreted by the cells of the innate immunity (epithelial cells, antigen-presenting cells, *et cetera*). Correspondingly, cytokine-related mechanisms are “switched on” in the earliest stages of the *Cryptosporidium* infection. Further on, antigen-activated CD4⁺ T-helper cells (as part of the adaptive immunity) also produce a diverse cocktail of cytokines related to the cell-mediated (IFN- γ , IL-2, and IL-12) and antibody-mediated (IL-4, IL-5, IL-6, and IL-10) immune responses.

IFN- γ (involved in both innate and acquired reactions) plays the most important role in the suppression of *Cryptosporidium* reproduction and the regulation of the parasitic infection (4, 5, 7, 10, 20, 27, 28). One of the most severe and ultimately fatal cases of cryptosporidiosis has been described in a child with a congenital deficiency in the synthesis of IFN- γ (29). Various experiments in transgenic murine lines confirm its

extreme importance. *Cryptosporidium* infection in IFN- γ -deficient mice results in persistent diarrhoea, which is even more severe than in CD4-knockouts (24). In other experiments, administration of IFN- γ -neutralizing antibodies to mice with SCID exacerbates the infection with *C. parvum* and reduces their survival significantly (23, 30). A substantial correlation exists between the kinetics of IFN- γ and the course of the acute cryptosporidiosis. IFN- γ levels rise and reach a peak in parallel with the clinical symptoms, and decline sharply with the onset of recovery (8). Despite the crucial role of IFN- γ in the early stages of immunological defence, most recent studies conclude that the ultimate resistance against cryptosporidiosis requires IFN- γ as well as normally functioning CD4⁺ T-lymphocytes (4, 7). Thus, IFN- γ probably exerts its protective effects against cryptosporidiosis in the domain of the acquired immune responses.

Some authors even provide evidence for the existence of alternative (IFN- γ -independent) pathways. Those are usually associated with increased production of IL-15 by the epitheliocytes of *Cryptosporidium* infected individuals (30). IL-15 levels correlate inversely with the parasite propagation. In vitro studies observe that IL-15 activates NK cells to eliminate intestinal *Cryptosporidium* infected epithelial cells (31). Neither IFN- γ nor IL-15 was detected in the intestinal epithelium of HIV⁺ patients with chronic and uncontrolled cryptosporidiosis (32). The relevant conclusion is that there are two separate innate (associated with IL-15) and adaptive (associated with IFN- γ) immune responses involved in the resistance against cryptosporidiosis (7, 10).

IL-12 (major inducer of IFN- γ synthesis) also plays a central role in the acquired and innate protection. Experimental studies demonstrate that treatment of newborn immunocompromised mice with IL-12 before infection creates resistance to cryptosporidiosis while treatment with IL-12-neutralizing antibodies increases the severity of the disease (33).

Conflicting results follow the investigations on the role of IL-4. After stimulation with *Cryptosporidium* antigens, human peripheral mononuclear cells demonstrate significant expression of both IL-4 and IFN- γ (34). Several contradictory hypotheses suggested that IL-4 is either: (i) involved in the late stages of the infection; (ii) promotes DCs activation; (iii) facilitates the protective Th1 reactions; (iv) plays part in the immune memory or (vi) has suppressive or no role whatsoever in the immunity against cryptosporidiosis (4, 6).

The protective effect of another cytokine, IL-5, is confirmed by an experiment in which the individual classes

of interleukins are blocked separately, and the authors account for the number of *Cryptosporidium* oocysts released from the infected animals. Highest levels of oocysts' production are reported in the experiments where both IL-4 and IL-5 were inactivated, demonstrating their synergistic effect in limiting cryptosporidiosis (35).

Numerous studies in mice and humans confirm the increased production of TNF- α after the infection of the intestinal mucosa with *C. parvum* (6, 36). Mutant murine lines with reduced intestinal TNF- α production cannot limit cryptosporidiosis compared to wild controls, and the supplementation of this cytokine leads to a rapid resolution (36). The protective effect of TNF- α is evident from several studies in which the addition of TNF- α to *C. parvum*-infected cell cultures of human and mouse enterocytes rapidly suppresses the parasite reproduction (27,37).

Other proinflammatory cytokines (IL-1, IL-6) released by multiple immunocompetent cells exert protection even in the absence of TNF- α (6). In mouse and human models of *Cryptosporidium* infection, IL-1 levels rise significantly. Additionally, *in vitro* experiments have confirmed its role as an inhibitor of the parasite reproduction in human enterocyte cultures (27, 37).

CYTOKINE-MEDIATED REACTIONS

The proinflammatory IFN- γ and TNF- α initiate the pathogen destruction via the generation of toxic nitric oxide (NO) by-products and oxygen radicals. Both cytokines (produced by the epithelial cells, active T-lymphocytes and NK cells), start the cascade of inducible nitric oxide synthase (iNOS) leading to the metabolism of arginine into NO and citrulline. In an experiment with athymic nude mice artificially supplemented with arginine, the faecal oocyst shedding is significantly lower compared to controls on a regular diet (38). The synthesis of NO also stimulates the expression of prostaglandin E₂, which improves the barrier function of epitheliocytes by increasing their electrical resistance (39). Since the iNOS is activated very early during infection, this process should be considered (at least partially) an innate immune mechanism. While several authors confirm the role of nitrogen derivatives in the *Cryptosporidium* destruction, other studies demonstrate inconclusive or contradictory results (27, 37).

The *Cryptosporidium* reproduction may be contained via additional IFN- γ induced mechanisms like the microenvironment depletion of essential metabolites (e.g. tryptophan or cellular iron) (27, 28). The direct effects of

IFN- γ on the intestinal epithelium may further inhibit the parasite invasion and its intracellular development (27). This vital mechanism of resistance to cryptosporidiosis is, however, still under investigation.

Many contemporary studies are trying to evaluate the involvement of other pro-inflammatory (IL-8, IL-18) and anti-inflammatory (IL-10, TGF- β) cytokines (4, 7, 10). Probably the resistance and the susceptibility to *Cryptosporidia* depends on a still to be revealed refined equilibrium between the synthesis of Th1-cytokines, involved in the control of the pathogen propagation, and Th2 factors directing the pathological process in the intestinal mucosa.

THE ROLE OF THE ADAPTIVE IMMUNITY

A variety of studies have shown that both cellular and humoral acquired immunity play a significant role in the control of cryptosporidiosis. Many of the reports are evaluating the severity of the clinical symptoms, oocyst production levels and the outcome of the cryptosporidiosis in animal (murine) lines with selected mutations leading to singular or combined T-cells deficiencies. The observation of the same parameters in the clinical cases of patients with AIDS and other types of primary immunodeficiencies may confirm the conclusions from animal testing but sometimes raises even more questions.

Numerous experiments have demonstrated that infection of *C. parvum* and *C. muris* elicits an antigen-specific immune response in the T-lymphocytes. The proliferation and cytokine production of lymphocytes from the spleen, MNs or peripheral blood from previously infected animals commence rapidly when the former are cultured with *Cryptosporidium* oocysts of the specific species (40, 41). Studies of immuno-naïve SCID mice show similar results. The immuno-deficient mice manage to fight off *C. parvum* or *C. muris* infection after the transfer of different T-lymphocyte populations from immunocompetent (pre-infected with the same species) animals (42).

Histological studies in healthy volunteers, patients with AIDS and cryptosporidiosis and experimental models have shown that the *C. parvum* infection leads to an upsurge in the number of T-lymphocytes subsets in the intestinal mucosa (43, 44) and substantial intensification in the production of proinflammatory cytokines (36). As cryptosporidiosis subsides, the number of T-cells in the intestinal lining declines to the normal levels (6). The first study to demonstrate the essential role of T-cells in the control of *Cryptosporidium* infection is in athymic mice (45). In the wild neonate animals, cryptosporidiosis was acute but rapid and self-limiting. In

T-cell deficient mice, on the other hand, the disease took a chronic course and was fatal in many cases (45). Similar persistent infection was demonstrated in the first experiments with SCID mice infected with *C. parvum* and *C. Muris* (17, 19). Furthermore, the experimental depletion of T-lymphocytes prevented recovery from *Cryptosporidium* infection in immunocompetent mice (4).

Countless epidemiological studies of cryptosporidiosis in humans confirm that the incidence of cryptosporidiosis with a diverse and deteriorating clinical presentation is higher in immunosuppressed patients (either suffering from a viral infection like HIV or due to immunosuppressive chemotherapies for a malignant process, or bone marrow/solid organ transplantation). In such cases, the diarrhoea persists for months, and the infection can involve extraintestinal sites (bile ducts, pancreas, respiratory system) and is lethal in some cases (1,4,25).

The utmost association of chronic and life-threatening cryptosporidiosis, usually with hepato-biliary involvement, is observed in patients with X-linked hyper-IgM syndrome. This primary immunodeficiency is caused by a specific mutation in one of the costimulatory molecules (CD40L) of the T-cells that activate IL-12 production by antigen-presenting cells and B-cell differentiation (1, 46, 47). Other primary immunodeficiencies that are susceptible to *Cryptosporidium* infection include primary CD4+ lymphopenia, IFN- γ deficiency and severe combined immunodeficiency syndrome (5).

Extensive research in the previous decades has shown that the control of the cryptosporidiosis in humans depends on the lymphocytes with the $\alpha\beta$ -form of T-cell receptors while the role of the T-cells with $\gamma\delta$ -receptors (even though they are in higher number in the mucosa) is secondary or even irrelevant (6). Additionally, the properly functioning subset of CD4+ lymphocytes is essential for the resistance to the *Cryptosporidium* infection. CD8+ T-cells are subordinate and play minor regulatory roles (1, 6, 42).

One of the main pieces of evidence for the fundamental role of CD4+ T-lymphocytes in the resistance against cryptosporidiosis is the increased susceptibility to the disease and its severe forms in patients with active AIDS, in whom the specific pathogenic effect of the HIV-virus significantly reduced the CD4+ cell population. Cryptosporidiosis usually is a self-limiting infection, even in HIV+ patients, when the CD4+ T-cell count exceeds 180 cells/l. If CD4-cell levels reach 100 cells/l cryptosporidiosis takes a chronic course and becomes fulminant, atypical and lethal when counts drop

below 50 cells/l (1, 7, 48, 49). Moreover, since the start of the application of the HAART, that leads to the restoration of CD4+ cell levels, cryptosporidiosis cases in this group have declined significantly, and even if the infection occurs, it is controlled very swiftly (1, 4, 7, 25, 50). This phenomenon directly correlates with the increase of the number of CD4+ T-lymphocytes in the intestinal mucosa (25).

The earliest experimental immunological studies of the disease demonstrate the critical role of the CD4+ cells in the course of the *Cryptosporidium* infection. In immunocompetent mice infected with *C. parvum* or *C. muris* inoculation with anti-CD4 antibodies depleting the CD4+ T-lymphocyte population, leads to clinical deterioration (42). On the contrary, in SCID mice, the inoculation with lymphocytes from immunocompetent animals provides adequate resistance to the infection. The disease, however, is not limited if the introduced cell line does not contain CD4+ T-cells (18, 42).

CD8+ T-cell numbers also increase in the affected intestinal sections. Their role is still unclear, and several experiments show that they are not essential for the resistance against the *Cryptosporidium* infection. In murine lines with MHC-I deficit and total lack of CD8+ T- lymphocytes, the elimination of *C. parvum* is commensurable with the observations in wild controls without immunodeficiency (51). In *C. muris*-infected mice, inoculation with anti-CD8 antibodies facilitates the parasite reproduction but not as much as when anti-CD4 antibodies are used (42). The current consensus is that CD8+ T-lymphocytes probably participate in the resistance against *Cryptosporidium* infection, but their role is regulatory and supplemental (4, 7)

Several recent reports, however, cast doubt on the significant protective impact of CD4+ T-cells against cryptosporidiosis, especially in newborns. In neonate murine models no considerable growth in the levels of CD4+ (and CD8+) T-cells was observed during *C. parvum* infection, neither in the Peyer's patches nor in *lamina propria* (52, 53). Furthermore, treatment of newborn mice with anti-CD4 neutralising antibodies had no notable effect on the susceptibility to infection (53). These findings support the concept that the processes of innate and adaptive immunity in the course of cryptosporidiosis are age-dependent as demonstrated by the highest frequency and severity of the disease in animal and human newborns alike (1, 54).

The evidence for the importance of humoral immunity for the control of *Cryptosporidium* infection is contradictory. Biopsy materials from experimentally infected volunteers

give evidence that B-lymphocytes are one of the first cells recruited to the site of infection. In lamina propria, their amount increased significantly as early as day 3, and antibody-producing B-cells appeared in the peripheral circulation on day five and remained for several weeks (7). Regrettably, most of the experiments on B-cell deficient murine lines show that, despite the rapid activation of these lymphocytes, the antibodies play a relatively small role in eliminating the infection (6, 7). There is not a significant difference in the course of cryptosporidiosis in SCID mice that underwent immune reconstruction with non-B-cell mucosal lymphocytes, compared to the lines where the recovery was achieved with lymphocytes from all cell populations (42). Similar results are reported in transgenic mice with congenital absolute B-cell deficiency where the severity of the disease and the time to elimination of the cryptosporidiosis are not substantially different as compared to the healthy controls (55).

The B-cells of the infected organism synthesise serum antibodies against various soluble membrane-associated proteins of the *Cryptosporidium* sporozoites - IgG (against 17-, 27-, and 15-kDa proteins), IgM (against 27-kD antigens) and IgA (predominantly against 17 kD proteins) (7, 56). The immunoglobulins are not species-specific but target *Cryptosporidia* of the entire genus and as proven by Priest *et al.* (57) in healthy volunteers infected with *C. hominis*, *C. parvum*, *C. felis* and *C. meleagridis*.

Various studies on the immune response and seroconversion in cryptosporidiosis demonstrate that increased levels of IgM and IgG synthesised in the previous infections have a partial protective effect. They mainly prevent the development of disease with pronounced clinical features but do not counteract the re-infection. Other experiments have linked the presence of IgG antibodies from previous exposure with the reduction of oocysts shedding upon re-infection (58, 59). The protective effect of the immunity developed after the initial infection is also indirectly confirmed by a majority of serological epidemic studies. These demonstrate that local population in certain regions suffers less from cryptosporidiosis in comparison to new arrivals, the elderly less than children, and the ones who consume insufficiently purified water, less than those with access to effectively disinfected water sources (58–60).

Mucosal IgA is considered the lead factor in protecting epithelial tissues against microbial agents. In murine experimental studies, Albert *et al.* (61) manage to transfer passive protection against *C. parvum*, by inoculating immunodeficient lines with bile derived IgA from self-cured

animals. Partial protection in newborn mice against *C. parvum* is demonstrated after inoculation with monoclonal IgA from B-lymphocytes from previously infected animals (62). IgA may contribute to the cellular response by coating the surface of the sporozoites and merozoites and averting the attachment and penetration into the epithelial cells (5). Several clinical cases of severe and persistent diarrhoea in patients with a congenital selective deficiency in the IgA-synthesis confirm its importance in the defence against cryptosporidiosis (63, 64).

The results from studies that evaluate the role of passive immunity in *Cryptosporidium* infection are inconsistent. Experiments with newborn calves confirm the passive transmission of antibodies and reduced severity of diarrhoea and parasite reproduction after oral administration of concentrated serum containing anti-*Cryptosporidium* antibodies (3). This is especially true when the experiments are conducted with hyperimmune colostrum derived from animals previously injected with purified oocyst antigens (3). Some epidemiological studies in humans suggest that cryptosporidiosis is less common in breastfed infants (65, 66), but others do not observe significant differences (67).

Overall, the results regarding the protective role of the B-cells and associated humoral immune response in the presence of *Cryptosporidium* infection are contradictory. The specific antibodies may have some (however minimal) capacity to reduce parasitic reproduction, but do not play a significant role in the immunological control of the disease and do not prevent re-infection. This results in another critical problem in the clinical practice: the serological tests are irrelevant and not useful for the routine diagnosis of cryptosporidiosis.

CONCLUSION

The immune response against infection by *Cryptosporidium* spp. includes all strata of innate and adaptive immunity with differences in their significance. The innate mechanisms of the intestinal mucosa (mucosal immunity), expressed predominantly by the “sentinel” role of epitheliocytes, are fundamental to the defence against this disease (Fig. 1). The vast array of epithelial chemokines and cytokines initiate the local inflammatory processes, attract other effector cells and directly inactivate the parasite adhesion. The second line of defence includes IFN- γ -production by the NK cells in combination with their innate cytotoxicity against the parasite and the infected epitheliocytes. The next level of resistance is the cell-mediated immunity with the significant participation of CD4⁺ T-lymphocytes, aided and regulated to some extent

by CD8⁺ cells. IFN- γ and other proinflammatory cytokines act directly on the infected enterocytes or activate a variety of immunocompetent cells. Even if some of the following effects are explained and experimentally proven, the vast amount of the interaction between mediators and cells is still under research. *Cryptosporidium* infection subsequently leads to the synthesis of specific serum and mucosal antibodies by the B-cells, but for now, the humoral immunity is considered to be of secondary and minor importance.

Despite the difficulties in conducting experimental studies in the fields of *Cryptosporidium* immunology (working either with cell cultures or with animal models) their realisation and implementation in the future is scientifically justified and of great practical importance. While the infection with *Cryptosporidium* spp. in immunocompetent individuals is mainly mild and asymptomatic, their role as one of the major opportunistic pathogens causing severe to life-threatening diarrhoea will remain essential, both in children and in the continually expanding group of patients with immunodeficiencies and systemic chronic diseases. Also, these studies reveal essential data about some still poorly understood mechanisms of mucosal immunity. These findings can further help to decipher the multicomponent immunological responses against other intestinal pathogens and reveal some of the pathogenetic mechanisms of autoimmune diseases of the intestinal mucosa.

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CONFLICT OF INTEREST

None to declare.

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