Review

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Host Responses to *Cryptosporidium* Infection

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Cryptosporidium is a clinically and economically important infection whose pathogenic effect begins with colonization of the intestinal epithelium. Despite intensive efforts, a consistently effective therapy for the infection has yet to be identified. Morbidity and mortality results from ongoing loss of absorptive epithelium, which leads to villous atrophy and malabsorption and release of inflammatory mediators that stimulate electrolyte secretion and diarrhea. With further clarification of the mechanisms underlying enterocyte malfunction in *Cryptosporidium* infection, it should be possible to design rational nutritional and pharmacologic therapies to enhance nutrient and water absorption, promote the clearance of infected enterocytes, and restore normal villus architecture and mucosal barrier function.

Key words: Cryptosporidiosis; Diarrhea; Immunity; Intestinal epithelium; Pathogenesis.

The single-columnar epithelial lining of the small intestine is the 1st line of defense against translocation of luminal bacteria, antigens, or endotoxin into the body while also being responsible for selective absorption of the majority of nutrients, electrolytes, and water required for life. These absorptive and barrier functions may be particularly compromised by infectious enteropathies in which the epithelial cells are the primary target of injury. *Cryptosporidium* is a highly infectious, epitheliotrophic intestinal pathogen that is resistant to many disinfectants, is small and difficult to filter, and is ubiquitous in many animals and the environment.[1,2] It can be the cause of severe, life-threatening diarrhea in HIV-infected people and is a leading cause of persistent diarrhea and infant mortality worldwide.[2,3] *Cryptosporidium* is considered a major threat to the US water supply, having been responsible in 1993 for the largest waterborne outbreak of diarrhea in US history.[4] Domestic animals serve as an important reservoir for environmental contamination and human infection, and cryptosporidial diarrhea accounts for the majority of economic losses suffered by the pork and dairy industries.[5–7] Currently, there are no consistently effective treatments for *Cryptosporidium* sp. infection or a number of other infectious enteropathies. With further clarification of the mechanisms underlying enterocyte malfunction in *Cryptosporidium* infection, therapeutic approaches designed to enhance nutrient and water absorption, parasite clearance, and epithelial repair are likely to diminish the morbidity, mortality, and economic impact of this as well as other epithelial pathogens.

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Infection of the Enterocyte by *Cryptosporidium*

Cryptosporidium has a complex life cycle that is normally confined to the intestinal epithelium of the host (Fig 1). Biliary, pancreatic, and respiratory epithelial involvement is also seen in some people with congenital (eg, X-linked hyper-IgM syndrome) or acquired (HIV) immunodeficiency and in some immunodeficient mouse models of the infection.[8–12] After ingestion, oocysts rupture under the influence of pancreatic enzyme activities and bile salts and release infective *sporozoites* into the lumen of the small intestine. Motile sporozoites adhere to absorptive villus epithelial cells, where they become enveloped by the apical enterocyte plasma membrane as *trophozoites.* Trophozoites proliferate asexually (merogony) to produce type I *meronts,* which contain 6–8 *merozoites.* Released merozoites infect additional enterocytes to form type I or type II meronts, the latter of which contain 4 merozoites. Merozoites released from type II meronts infect additional enterocytes and proliferate sexually (gametogeny) to form either a male *microgamont* or a female *macrogamont.* Microgametes released from the male microgamont fertilize the female macrogamete to form a *zygote.* The zygote undergoes meiosis (sporogony) to form an *oocyst* containing 4 sporozoites. Two types of oocysts are formed—thin-walled oocysts that can rupture in the intestinal lumen and immediately reinfect the epithelium (autoinfection) and thick-walled oocysts that are excreted in the feces and are immediately infective when ingested.[13,14]

Surprisingly, little is known about the direct effect of *Cryptosporidium* on the parasitized epithelial cell. Attachment of sporozoites to the apical plasma membrane of the enterocyte appears to be prerequisite to the pathophysiologic sequelae of infection insofar as *Cryptosporidium*-conditioned media or heat-inactivated organisms fail to reproduce the clinical manifestations of disease.[15,16] Details regarding the interactions between host enterocyte and parasite are poorly understood. The sporozoite attaches by its anterior pole to the apical membrane of the enterocyte, and antibodies recognizing glycoprotein antigens on the sporozoite surface can inhibit parasite attachment.[17–20] Recognition of specific ligands on the apical enterocyte membrane is suggested by the inability of trophozoites to infect the basolateral membrane, even in cultured epithelia.[21] After attachment, there is focal disassembly of the microvillous

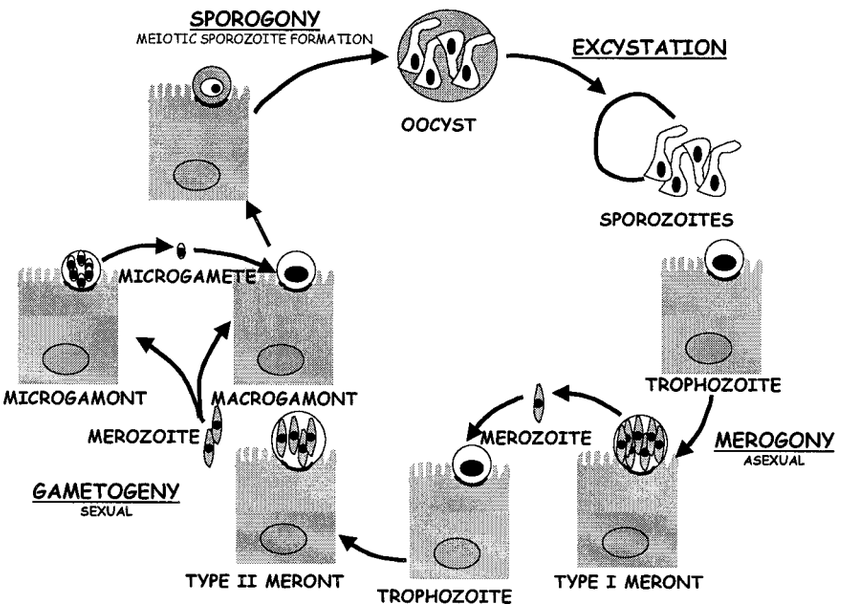

Fig 1. Life cycle of intestinal *Cryptosporidium* infection.

brush border and invagination of the enterocyte membrane, which engulfs and eventually surrounds the parasite to form a parasitophorous vacuole. Within this vacuole, the organism is both intracellular and extracytoplasmic.[15,17,22] This unusual location may provide an important barrier to the access of antimicrobial agents to the organism.[23] The parasite remains separated from the enterocyte cytoplasm by an attachment zone of extensively folded membrane referred to as the ''feeder organelle.'' Ultrastructurally, this parasite-enterocyte interface exists as an electron-dense band of cytoskeletal and intracellular signaling proteins.[24–27] Rearrangement of the actin cytoskeleton at this interface appears to be required for *Cryptosporidium* infectivity.[26]

Mechanisms of Epithelial Injury in *Cryptosporidium* Infection

Numerous observations suggest that *Cryptosporidium* is directly injurious to the intestinal epithelium. Foremost of these observations is the presence of severe villous atrophy in animals with active infection (Fig 2).[28] Villous atrophy is the reduction in villous surface area that results from ongoing loss of surface enterocytes. This ongoing loss is compensated for by hyperplasia of the crypt epithelium, which provides replacement enterocytes to the villus. Secondly, *Cryptosporidium* infection is associated with an increase in transepithelial permeability.[15,21,29,30] In people with HIV-related cryptosporidiosis, in vivo intestinal lactulose and mannitol permeability are increased.[31] Some studies have reported a decrease in in vitro mucosal permeability after *Cryptosporidium* infection.[32,33] Importantly, these studies do not account for the diminished surface area of infected mucosa, which is a consequence of the severe villous atrophy. When in vitro measurements of permeability are calculated with respect to the actual mucosal surface area present, increased epithelial permeability is disclosed (Gookin and Argenzio, personal communication). In neonatal pigs and calves, infection with *Cryptosporidium* occasionally results in gross epithelial disruption.[28,32]

Although epithelial damage is clearly a consequence of *Cryptosporidium* infection, what remains less certain are the precise mechanisms involved and the relative role of the organism versus the host response in creating the injury. *Cryptosporidium* may cause enterocyte injury by several potential mechanisms, including a direct cytotoxic effect, induction of apoptosis of the host enterocyte, or by initiating a change in phenotype of the enterocyte, which targets its elimination by innate or specific immune mechanisms.

Direct Cytotoxicity

Evidence for a direct cytopathic effect of *Cryptosporidium* is surprisingly limited and is based on studies of infected intestinal epithelial cell cultures. These studies have shown that infection of an epithelial monolayer results in leakage of the cytosolic protein lactate dehydrogenase (LDH) into the culture medium.[15,16,29] Release of LDH correlates with the dose of oocysts and accumulates only within the media bathing the apical side of the infected monolayer. Other studies have demonstrated selective accumulation of propidium iodide across the apical, but not the basolateral, membrane of infected epithelial cells. These findings suggest that *Cryptosporidium* disrupts the apical membrane of the host enterocyte, an effect that may be linked to function of the feeder organelle.[29]

Apoptosis

In contrast to cytotoxic effects, studies of *Cryptosporidium*-infected biliary[21,34] and intestinal epithelial cell cultures[35] suggest an important role for apoptosis in mediating epithelial injury. Apoptosis is a form of cell death in which the cell activates its own internal death program. There is a dose- and time-dependent increase in number of apoptotic cells within the infected monolayer. In biliary epithelia, in-

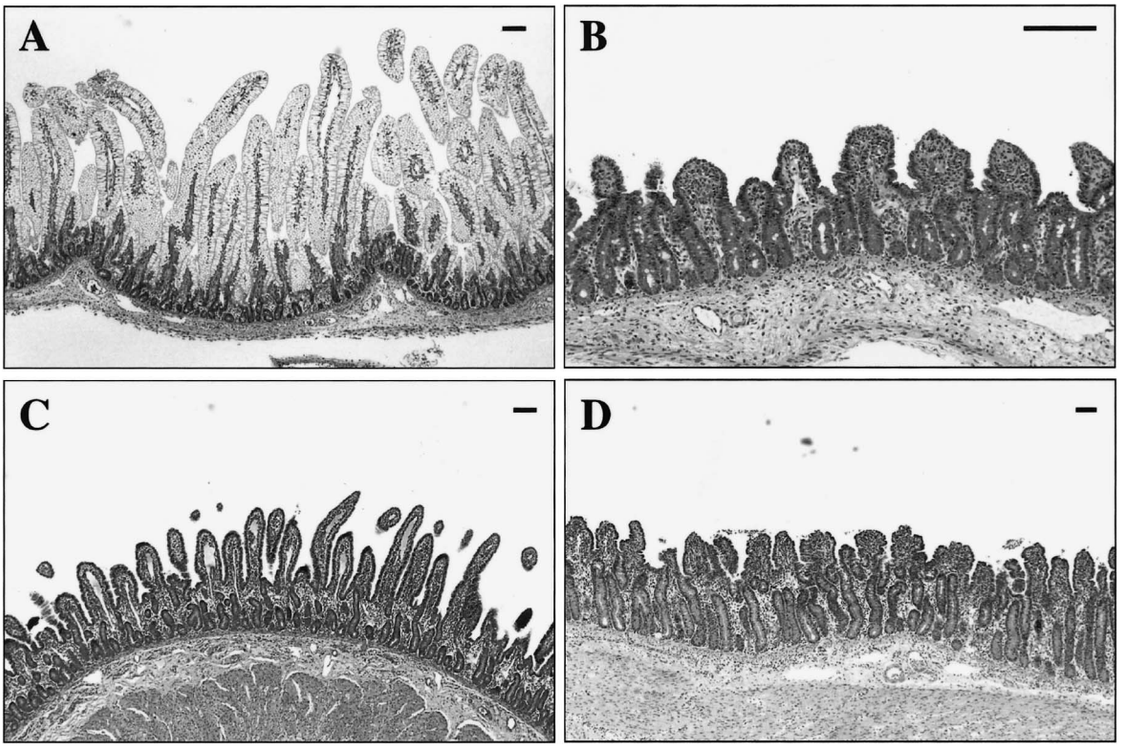

Fig 2. Normal and *Cryptosporidium parvum*-infected mucosa from neonatal pig and calf ileum. **(A)** Normal neonatal piglet ileal mucosa is greatly amplified by tall villous projections. In neonates, these villi are lined by foamy enterocytes that are specialized for pinocytosis. Magnification 16×. **(B)** Ileal mucosa from a neonatal piglet experimentally infected with *Cryptosporidium parvum.* Epithelial infection has resulted in loss of surface enterocytes and severe villous atrophy. Magnification 50×. **(C)** Normal neonatal calf ileal mucosa. Villous projections appear to lack foamy enterocytes (an observation of undetermined significance). Magnification 16×. **(D)** Ileal mucosa from a neonatal calf experimentally infected with *Cryptosporidium parvum.* There is villous atrophy and epithelial disruption. Hyperplasia of crypt epithelium provides replacement enterocytes to the villus. Note the relatively mild degree of inflammatory cell infiltration of both the piglet and calf lamina propria. Magnification 13.2×. Hematoxylin and eosin stain. Bar = 200 μm.

fection results in the synthesis and release of Fas ligand into the culture medium while at the same time stimulating surface expression of its transmembrane receptor protein Fas.[34] Activation of Fas by Fas ligand results in activation of the death program. These studies also demonstrated that Fas ligand mediates apoptosis of uninfected enterocytes as well, thus contributing to a nonselective epithelial injury. Although apoptosis can lead to death of the infected enterocyte, there is evidence that *Cryptosporidium* subverts this host attempt to eradicate infection. For example, after infection of intestinal epithelial monolayers, apoptosis is restricted to cells containing the parasite, but the majority (80%) of infected cells present are not apoptotic.[35] Protection from apoptosis appears to result from parasite activation of the transcription factor NF-κB.[35] The extent to which apoptosis contributes to enterocyte losses in *Cryptosporidium* infection and the specificity of this mechanism in vivo await the results of further study.

An alternate hypothesis to targeted apoptosis of infected cells is the possibility that enterocyte losses result from an acceleration of the normal program of epithelial turnover, in which enterocytes produced in the crypt are eventually terminated by apoptosis at the villus tip. Such a hypothesis would be consistent with the commonly observed lack of disruption of infected epithelia in vivo. In support of this hypothesis is a study of porcine ileal *Cryptosporidium* infection in which 15% of infected enterocytes exfoliated from the side of the villus, whereas 85% were extruded at the villus tip.[32]

Mucosal Inflammation

Cryptosporidium infection of epithelial cell cultures and xenografts of human intestinal mucosa result in the polarized secretion of neutrophil chemokines and activation factors (IL-8, GROα, IL-1β, and TNFα) from the basolateral surface of host enterocytes.[16,36] Cytokine release is in direct proportion to the number of infecting organisms.[16] Experimental *Cryptosporidium* infection of neonatal pigs results in a significant influx of neutrophils and macrophages and increased concentrations of malondialdehyde (a product of lipid peroxidation).[28,37,38] This occurs within the lamina propria at the peak of infection and correlates directly with the number of parasitized enterocytes and degree of villous atrophy.[28] There is no change in the total number of cells within the lamina propria surrounding the crypts, suggesting that a greater concentration of inflammatory cell mediators is brought to bear on infected villus rather than crypt enterocytes.[28] Despite these observations, inflammatory cell infiltrates are often mild in *Cryptosporidium* infection, sug-

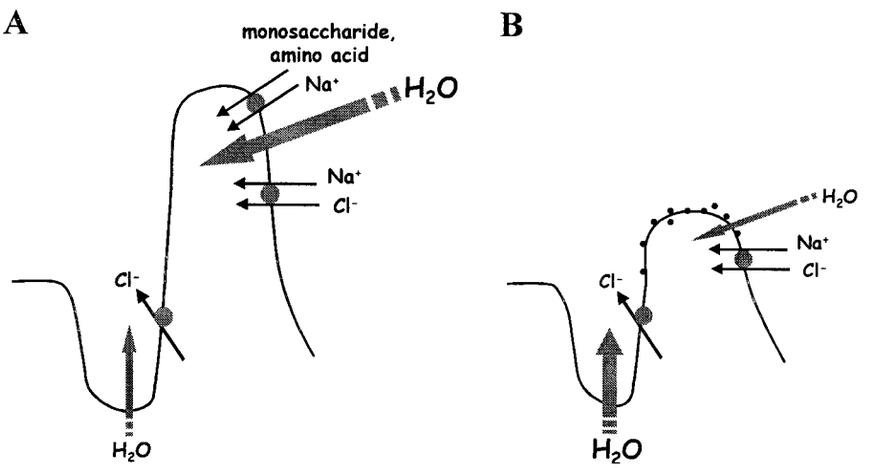

Fig 3. Consequences of villous atrophy in *Cryptosporidium* infection. **(A)** Net movement of water across the small intestinal mucosa is determined by the balance between villous absorption and crypt secretion. Absorptive transport mechanisms are expressed by mature villus enterocytes and include nutrient-coupled Na^+ transporters and neutral NaCl transporters. **(B)** In *Cryptosporidium* infection, loss of villus enterocytes results in severe villous atrophy with nutrient and electrolyte malabsorption and shifts water balance in favor of net secretion.

gesting that they are unlikely to be a primary cause of the epithelial cell losses.

Mechanisms of Diarrhea in Cryptosporidium *Infection*

The pathophysiology of *Cryptosporidium*-associated diarrhea is complex. Diarrhea appears to be primarily a consequence of (1) severe villous atrophy, which diminishes absorption, and (2) altered electrolyte transport, which results from the release of inflammatory mediators. Secretion of an enterotoxin by *Cryptosporidium* is suggested by some studies[39]; however, this remains controversial. Fluid absorption by the small intestine is the net result of nutrient-coupled Na^+ and NaCl-absorptive processes on the villus and anion secretory mechanisms in the crypts. The villus absorptive processes are thought to be expressed only by the most mature enterocytes at the villus tip. Accordingly, part of the fluid losses in *Cryptosporidium* infection are believed to be a direct consequence of villous atrophy and the associated electrolyte and nutrient malabsorption (Fig 3). For example, impaired glucose- and glutamine-coupled Na^+ absorption has been identified in piglet and rat models of the infection.[28,33,40–42] In people with HIV-related cryptosporidiosis, vitamin B_{12} and D-xylose absorption are diminished and correlate with the location of mucosal infection (ileum and proximal small intestine, respectively) and extent of villous atrophy.[31] It is probable that increased mucosal permeability also contributes to ineffective electrolyte and nutrient absorption in *Cryptosporidium* infection, although studies have not been performed to substantiate this.

Role of Endogenous Prostaglandin (PG) Synthesis

Nevertheless, an equally important loss of fluid in cryptosporidial infection has been shown to involve a PG-mediated alteration in electrolyte transport (Fig 4). Concentrations of the endogenous PGs, PGE_2 and PGI_2, are higher in infected tissue and inhibit NaCl absorption and induce anion (Cl^- or HCO_3^-) secretion.[41] These alterations are due both to direct effects of PGE_2 on the epithelium and indirect effects via PGI_2 activation of the enteric nervous system.[43]

The source of high PGs in infected tissue has not been definitively established but may be the result of infiltrating PMNs and macrophages,[28,37,41,43] whose products have been shown to strongly induce PG synthesis from mesenchymal cells in the lamina propria.[44] Conversely, in human intestinal epithelial cell cultures, *Cryptosporidium* directly activates PGH synthase 2 expression and PGE_2 synthesis by infected cells.[45] The relative contribution of these mechanisms to net PG production in vivo and the signaling pathways leading to altered electrolyte transport need to be resolved, as this may have important therapeutic implications.

Surprisingly, infected piglet ileum treated with the PG synthesis inhibitor indomethacin displays normal or even augmented rates of NaCl absorption despite loss of two-thirds of the villous surface area.[41] In the normal piglet ileum, the villous epithelium is highly vacuolated as a consequence of ongoing pinocytosis (Fig 2).[46] It is possible that these specialized cells do not normally contribute to NaCl absorption or that the NaCl transporter has been markedly up-regulated in the remaining epithelium. The latter is a distinct possibility, because glucocorticoids have been shown to induce NaCl transporter mRNA transcription paralleled by increased activity of this transporter in rat ileum and proximal colon.[47] Although nonselective PG synthesis inhibitors are capable of restoring normal NaCl absorption, their in vivo use in piglets with cryptosporidiosis results in increased synthesis of TNFα by the intestinal mucosa and more severe villous damage.[48] Surprisingly, little evidence exists either in support of or against the use of PG synthesis inhibitors in any experimental or natural infection of the intestinal epithelium. Selective PG inhibitors or inhibitors of upstream or downstream mediators of excessive PG synthesis may ultimately be most beneficial. For example, inhibitors of enteric cholinergic or vasoactive intestinal polypeptide-secreting (VIPergic) nerves, downstream effectors of PGI_2 production in the pig, attenuate the altered NaCl transport of the infection by some 50%.[43]

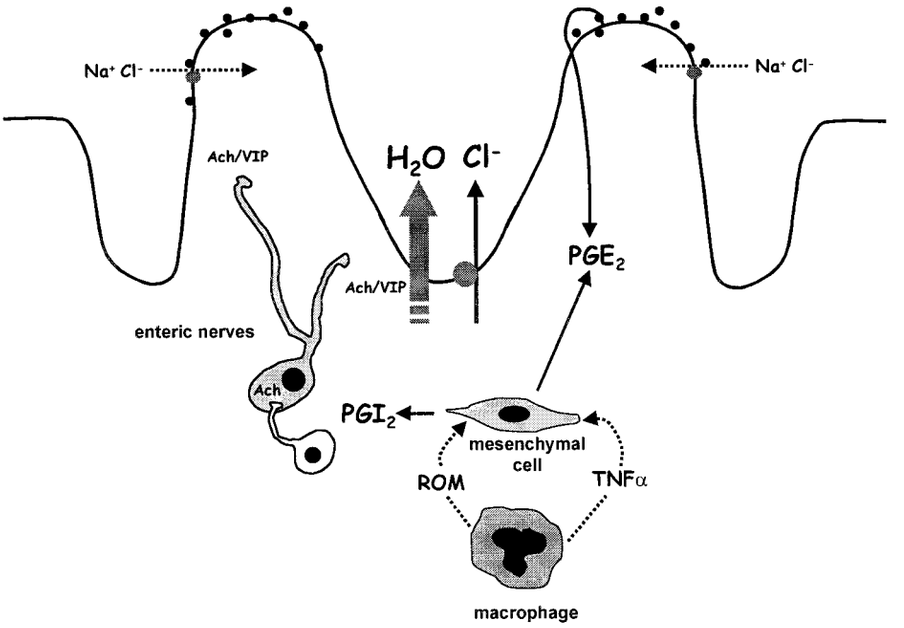

Fig 4. Role of endogenous prostaglandin (PG) synthesis in *Cryptosporidium* infection. Both PGI_2 and PGE_2 are increased in infected mucosa and result in altered electrolyte transport. PGI_2 is released from cells in the lamina propria in response to mucosal inflammation and stimulates cholinergic and vasoactive intestinal polypeptide-secreting (VIPergic) enteric nerves that lie in close proximity to the epithelium. Acetylcholine (Ach) and VIP alter electrolyte transport by increasing enterocyte cAMP and Ca^{++} 2nd messengers, respectfully. This results in stimulation of Cl^- secretion by crypt enterocytes and inhibits NaCl absorption by villus enterocytes. PGE_2 is synthesized by both the lamina propria and the infected epithelium. PGE_2 directly stimulates Cl^- secretion by crypt enterocytes and inhibits NaCl absorption by villus enterocytes by increasing enterocyte cAMP. ROM, reactive oxygen metabolites; TNFα, tumor necrosis factor alpha.

Mechanisms of Recovery from Cryptosporidium *Infection*

Adaptive immunity plays a pivotal role in determining the susceptibility to and ability to recover from *Cryptosporidium* infection. For example, infection in neonates is more common and severe than in adults, presumably because of incompletely developed adaptive immunity in neonates. Recrudescence of occult infection can be seen during immunosuppressive therapy, and the prevalence of infection is greater in people and animals with congenital and acquired immunodeficiency.[49–54] The critical mediators involved in recovery from *Cryptosporidium* infection appear to be T lymphocytes, the cytokine IFNγ, and intercellular communication, which depends on a transmembrane protein CD40 and its cognate ligand CD154.

T-Lymphocyte Response to Infection

Immunodeficient mouse models have provided considerable insight into the role of lymphocytes in protection and recovery from *Cryptosporidium* infection. T cells appear to be essential for this purpose. Severe combined immunodeficient (SCID) mice (lacking both T and B cells) and nude mice (lacking only T cells) develop chronic cryptosporidiosis after experimental infection, whereas mice lacking only B cells recover normally.[49–51,54,55] Adoptive transfer of $CD4^+$ lymphocytes (helper T cells) to SCID mice is markedly more effective at mediating clearance of infection than reconstitution with $CD8^+$ lymphocytes (cytotoxic T cells).[56] Further supporting these studies is the observation that αβ T-cell receptor $(TcR)^+$ lymphocytes are necessary for host control of cryptosporidiosis, whereas γδ TcR^+ cells are not.[57] The TcR is the extracellular molecule expressed on T cells that recognizes antigen associated with major histocompatibility proteins (MHC) of antigen-presenting cells. Most circulating $CD4^+$ T cells express αβ TcR, whereas the γδ TcR is usually expressed on intra-epithelial $CD8^+$ T cells of the gastrointestinal tract. The relative importance of $CD4^+$ versus $CD8^+$ T-cell populations to clearance of *Cryptosporidium parvum* infection has been further investigated with major histocompatibility antigen (MHC) class I and II deficient mice. MHC II-deficient mice lack functional $CD4^+$ T cells and develop severe and protracted infection after ingestion of *C parvum* oocysts, whereas MHC I-deficient mice, which lack functional $CD8^+$ T cells, do not.[58] In reconstituted SCID mice that have recovered from infection, donor lymphocytes have been demonstrated to migrate to the recipient's intestinal epithelium[54,59] and release IFNγ in the presence of soluble oocyst antigen.[54] These combined observations indicate a critical role for the $CD4^+$ T cell in recovery from *Cryptosporidium* infection.

Although the role of $CD8^+$ T cells in protection and clearance of infection in immunodeficient mouse models is weak, acute *C parvum* infection of neonatal calves is associated with dramatic increases in the number of intraepithelial $CD8^+$ T cells isolated from diseased ileal mucosa.[60,61] Species differences in the percentage of T-cell populations within the gastrointestinal tract may ultimately be found to contribute to species-specific mechanisms of clearance of *C parvum.* Lymphoid cells of the gut-associated

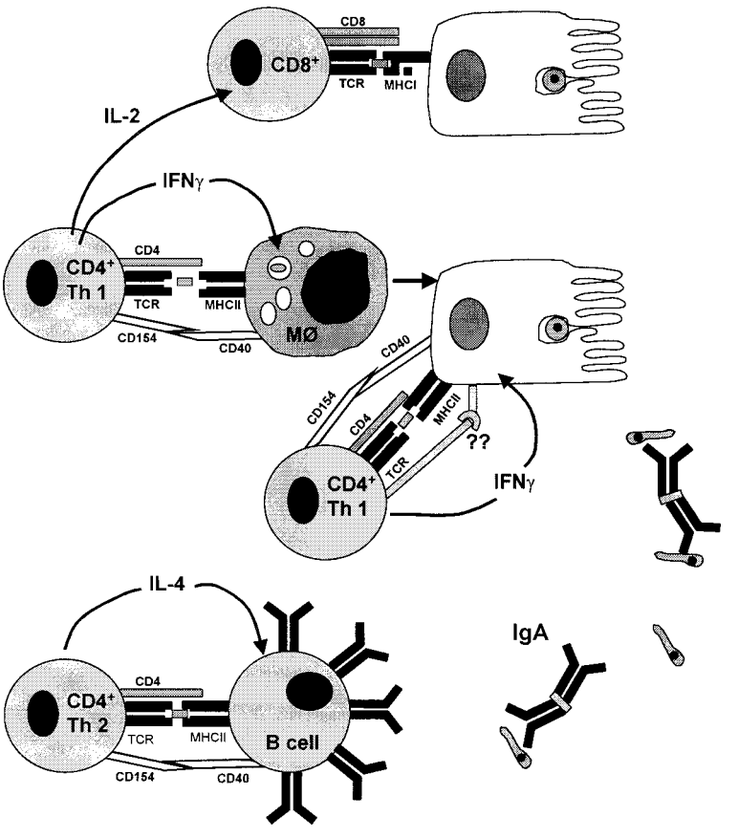

Fig 5. Immune responses to *Cryptosporidium* infection. T-helper lymphocytes ($CD4^+$) fall into 2 functionally distinct types based on the cytokines they secrete. Both types express T-cell receptors (TCR) that recognize antigen in the context of major histocompatibility complex II (MHCII) molecules. Th1-type cells secrete cytokines that activate cell-mediated immune responses. The cytokine interferon gamma (IFNγ) activates macrophages (MØ), and interleukin-2 (IL-2) results in proliferation of cytotoxic T cells ($CD8^+$). The specific antigen recognized by early Th1-type cells in *Cryptosporidium* infection is unclear, as is their role in elimination of infected enterocytes. Th1-type cells may directly interact with infected enterocytes or stimulate macrophage or cytotoxic T-cell ($CD8^+$) responses. Th2-type cells secrete cytokines that activate B lymphocytes and antibody (eg, IgA) synthesis. Neutralizing antibody promotes clearance and immunity to *Cryptosporidium* infection.

lymphoid tissue are normally found in 3 compartments: (1) the connective tissue of the lamina propria, (2) Peyer's patches, and (3) within the epithelium. Once within the epithelium, the intra-epithelial lymphocytes (IELs) are uniquely poised to execute immune system defense against mucosal pathogens.[53] Several studies have demonstrated an increase in numbers of IELs after *Cryptosporidium* infection[53,59–62] as well as an apparent physical association between the IELs and infected enterocytes.[53,61] Approximately 25% of peripheral blood lymphocytes and IELs of calves are γδ TcR^+ (see Waters et al[63]) and therefore may play a larger role in control of cryptosporidiosis in cattle than they do in mice. Collectively, these findings suggest the possibility of dramatic species differences in the type of immune response to *Cryptosporidium* infection.

Role of the $CD4^+$ Th1-Type Cell

Further investigation of the role of the $CD4^+$ T cell in recovery of mice infected with *Cryptosporidium* has revealed a biphasic response involving 2 functionally distinct $CD4^+$ T-cell subtypes.[64–69] These subtypes are identified by virtue of the complement of cytokines they secrete. Th1-type cells elaborate cytokines (eg, IFNγ and IL-2) that promote cell-mediated immune responses by phagocytes and cytotoxic T cells ($CD8^+$) (Fig 5). These IFNγ-secreting $CD4^+$ T cells increase early (day 9) in murine *Cryptosporidium* infection.[69] Cytokine depletion experiments and studies in mice genetically deficient in selected cytokines have shown that Th1 cells are critical for activation of protection and clearance.[64–68] Depletion of IFNγ or IL-12 by means of monoclonal antibodies increases the severity and duration of infection.[64,67,68] A single dose of recombinant IL-12, given before experimental infection with *C parvum*, has been shown to prevent infection of both immunocompetent and SCID mice.[68] However, exogenous IL-12 is not effective when given after the onset of infection. The action of IL-12 in preventing infection is believed to be through the induction of IFNγ, in that co-administration of anti-IFNγ and recombinant IL-12 negated the protective effect of recombinant IL-12.[68] In separate murine studies, neutralization of IFNγ with anti-IFNγ monoclonal antibodies enhanced oocyst shedding[64] and extended the period of oocyst excretion,[69] although infection remained self-limiting. It was only upon neutralization of both IFNγ and $CD4^+$ T cells that shedding was dramatically increased, and infection became chronic.[64] These observations suggest that both

$CD4^+$ T cells and IFNγ are required to prevent initiation of infection, with IFNγ playing a major role in limiting the severity of infection and $CD4^+$ T cells influencing the duration of infection.

The precise effector mechanism of the $CD4^+$ T-cell response is not known. When cultured ex vivo, these early-responding cells do not appear to specifically recognize *Cryptosporidium* antigen.[69] In addition, when $CD4^+$ T cells bearing a single specificity of T-cell receptor that recognizes only chicken ovalbumin are transferred into SCID mice, they migrate to the intestinal mucosa, become activated, and eliminate *Cryptosporidium*-infected enterocytes.[70] When these ovalbumin-specific $CD4^+$ T cells are re-isolated from the mice after infection, they do not proliferate in response to *C parvum* antigen exposure.[70] The role of the TcR in these responses is unknown, and it remains unclear how these lymphocytes recognize and promote clearance of infected enterocytes either through direct interaction with infected enterocytes or via activation of macrophages or cytotoxic $CD8^+$ T cells (Fig 5). The hypothesis that early-responding $CD4^+$ T cells might combat infection in a non-antigen-restricted manner is an unconventional one.

*Role of IFN*γ

Cryptosporidium infection is associated with increased synthesis of IFNγ both in vitro and in vivo.[11,68] Potential sources of IFNγ include the $CD4^+$ T cells themselves, as well as $CD8^+$ T cells and natural killer cells. Several observations suggest that IFNγ is a major effector cytokine of the immune response against *Cryptosporidium* infection. IFNγ knockout mice or mice treated with anti-IFNγ antibody are highly susceptible to *Cryptosporidium* infection[11,71,72] and have increased numbers of infected enterocytes and oocyst shedding.[11,68] Conversely, treatment with recombinant IFNγ diminishes the parasite load of infected intestinal epithelium.[11] Exactly where and how IFNγ mediates a decrease in number of infected enterocytes and oocyst shedding is unknown.

IFNγ has direct effects on epithelia, including induction of MHC I and MHC II receptors, expression of β_2 integrin-dependent epithelial ligand,[73] and up-regulation of transmembrane CD40 expression,[74] and triggers the opening of intercellular tight junctions.[30] These alterations may equip the enterocyte for interaction with cytotoxic effector cells of the specific and innate arms of the immune response. IFNγ also inhibits the ability of attached *Cryptosporidium* organisms to invade epithelial cells in culture[75] and induces nitric oxide (NO) synthesis.[76]

Role of CD154 Expression

The ligand CD154 is expressed predominantly by activated $CD4^+$ (T helper) lymphocytes.[77] The receptor for CD154 is called ''CD40'' and is an integral membrane protein that can be expressed by numerous cell types, including B lymphocytes, epithelial cells, and macrophages. Engagement of CD154 and CD40 can result in a myriad of responses from stimulatory to induced cell death. Such interactions appear to be required for elimination of *Cryptosporidium* infection.[10] For example, T cells deficient in CD154 fail to confer immunity when transferred to *Cryptosporidium*-infected SCID mice.[10] Also, children with a genetic deficiency in expression of CD154 (X-linked hyperimmunoglobulin M syndrome) are predisposed to chronic *Cryptosporidium* infection.[8] Whereas the $CD4^+$ T cell is the likely source of CD154 in *Cryptosporidium* infection, the CD40-bearing cell type engaged by CD154 remains uncertain. CD40 can be expressed in vitro by *Cryptosporidium*-infected bile duct epithelial cells and cultured hepatocytes,[10] and CD154 can mediate apoptosis of infected hepatocytes bearing CD40.[21] These observations suggest that $CD4^+$ T cells may interact directly with infected enterocytes via CD154-CD40 interaction (Fig 5). Potential consequences of this interaction include increased cellular NO synthesis[74,78,79] induced expression of Fas and Fas ligand, which initiate apoptosis, or direct activation of pro-apoptic intracellular signaling pathways (ie, caspases 8 and 3), which eliminate the infected enterocyte. Mice with Fas or Fas ligand deficiency are capable of recovering from infection, suggesting these molecules do not singularly affect clearance[80] (Perryman and Nordone, personal communication).

Role of NO

In mice experimentally infected with *Cryptosporidium*, inducible NO synthase enzyme (iNOS) is expressed by the infected epithelium, and plasma NO concentration is increased.[78] Several observations suggest that these increases in NO synthesis play a role in recovery from infection. Firstly, iNOS knockout mice and mice treated with iNOS inhibitors have increased susceptibility to *Cryptosporidium* infection, increased oocyst shedding, increases in epithelial colonization, and delayed parasite clearance.[78,79] Likewise, treatment of infected mice with L-arginine or an NO donor decreases epithelial infection and oocyst shedding.[78,78] Although contributing to elimination of *Cryptosporidium* infection, it appears unlikely that NO is an essential factor for recovery. For example, iNOS knockout mice and mice treated with iNOS inhibitors are capable of recovering normally from *Cryptosporidium* infection.[80,81]

The cellular source of NO and its precise role in mediating clearance of *Cryptosporidium* organisms and infected enterocytes is not known. NO inhibits the growth and function of numerous microbial pathogens.[82] In vitro, NO donors have been shown to inhibit excystation of *Cryptosporidium* sporozoites and to reduce sporozoite viability.[79] This mechanism likely involves the inactivation by NO of critical metabolic pathways mediated by Fe^{2+}-containing metalloenzymes of the organism.[82] Further, the infected enterocytes themselves may be an important source of NO, and IFNγ has been shown to stimulate high-output NO formation by cultured epithelial cells.[76] IFNγ is unlikely to mediate its effects entirely by stimulating NO synthesis, however, because IFNγ knockout mice have more severe disease than iNOS knockouts.[78] The ability of enterocytes to produce high NO concentrations may play an important role in mucosal defense against epithelial pathogens, either by injuring the parasite or by eliminating the infected enterocyte.[83] The unapposed generation of NO induces apoptosis, and, in the presence of superoxide, NO is converted to an extremely potent oxidant peroxynitrite.[84,85] Peroxynitrite may mediate the anti-cryptosporidial effects of NO as

treatment of *Cryptosporidium*-infected mice with antioxidants (ascorbic acid or superoxide dismutase) worsens oocyst shedding and enterocyte infection.[78]

Role of the CD4⁺ Th2-Type Cell

The resolution phase of murine *Cryptosporidium* infection (day 23) is accompanied by sustained increases in IL-4–secreting (Th2-type) $CD4^+$ T cells within the gastrointestinal mucosa.[69] These lymphocytes show specific responses to *Cryptosporidium* antigen when cultured ex vivo.[69] Th2-type cells elaborate cytokines (eg, IL-4 and TGFβ) that promote B cell activation and immunoglobulin synthesis (Fig 5). B-cell–deficient mice and mice treated with anti–IL-4 antibody demonstrate delayed but eventual resolution of infection.[55,69] That antibody synthesis is not required for recovery is typified by the normal serum and secretory antibody responses in people with HIV and chronic *Cryptosporidium* infection. Nevertheless, production of neutralizing antibody likely hastens recovery from infection by inhibiting the cycle of reinfection by intraluminal stages of the organism and plays an important role in protection from reinfection.

Experimental studies with genetically deficient murine models or neutralizing antibodies have simplified study design and have led to delineation of key cell types and cytokines involved in protection and recovery from *Cryptosporidium* infection. It is important to consider, however, that neutralization of IL-4 or IFNγ, for example, results in a compensatory increase in the alternate cytokine-expressing cell type. Indeed, an increased capacity to up-regulate IL-4 appears to be responsible in part for the ability of 1 strain of IFNγ knockout mice to recover from infection (BALB/c), whereas another strain cannot (C57BL/6).[11,86,87] No direct evidence exists for genetic susceptibility to *Cryptosporidium* infection. However, the wild-type mice (BALB/c and C57BL/6) used in the aforementioned studies differ in susceptibility to a variety of intracellular pathogens, even in the absence of deleted immune response genes. These differences have been related to the presence or absence of a functional resistance gene called *Ity*, which encodes a macrophage membrane transporter.[88] Genetic variants of *Ity* have been identified in humans and cattle.[88,89] Future research may ultimately demonstrate that during an immunosuppressed state, an individual's genetic makeup may influence the predisposition to or severity of *Cryptosporidium* infection.

From the aforementioned experiments, a model of epithelial recovery from primary *Cryptosporidium* infection can be developed wherein αβ $CD4^+$ T cells mediate a non-antigen-specific clearance of infected enterocytes. $CD4^+$ T cells could be recruited to infected intestinal epithelial cells and become activated within the proinflammatory environment, physically associate with epithelial cells in a CD40-CD154–dependent manner, and convey pro-apoptotic signals leading to eradication of the infected enterocyte. The fact that immunocompetent hosts are resistant to reinfection suggests that antigen-specific mechanisms do develop and are responsible for protection from subsequent challenge. Characterization of this secondary response has yet to be fully characterized.

Conclusion

Cryptosporidium is a clinically and economically important infection whose pathogenic effect begins with colonization of the intestinal epithelium. Despite intensive efforts, a consistently effective therapy for the infection has yet to be identified. Morbidity and mortality results from ongoing loss of absorptive epithelium, which leads to villous atrophy and malabsorption and release of inflammatory mediators that stimulate electrolyte secretion and diarrhea. With further clarification of the mechanisms underlying enterocyte malfunction in *Cryptosporidium* infection, it should be possible to design rational nutritional and pharmacologic therapies to enhance nutrient and water absorption, promote the clearance of infected enterocytes, and restore normal villus architecture and mucosal barrier function.

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