Cryptosporidiosis Induces a Transient Upregulation of the Oligopeptides Transporter (PepT1) Activity in Neonatal Rats

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Cryptosporidium parvum **is a parasitic protozoa increasingly appreciated as a cause of intestinal malabsorptive syndrome leading to malnutrition and/or growth failure. Because a major mechanism for apical peptide absorption by small intestine is *via* the proton-coupled transporter PepT1, we investigated the expression and functionality of this transporter in our model of acute cryptosporidiosis. Four-day-old Sprague-Dawley rats were inoculated by gavage with 5×10^5 oocysts of *C. parvum* and killed at Day 12 (peak of the infection) or Day 21 (spontaneous clearance of the parasite). PepT1 expression and functionality were quantified in the distal small intestine, preferential site of *C. parvum* implantation, and in the proximal small intestine, free of parasite, using Western blot and Ussing chambers, respectively. No difference in total PepT1 protein expression or in glycyl-sarcosine fluxes was observed in *C. parvum*–infected rats compared with controls either on Day 12 or on Day 21, both in the proximal and in the distal small intestine. However, a significant decrease of apical membrane protein expression of PepT1 was observed in *C. parvum*–infected enterocytes compared with controls. This maintained dipeptide transport observed despite villous atrophy and decreased expression of the protein at the brush-border membrane strongly suggest a transient upregulation of PepT1 activity, probably related to γ-interferon regulation.** Exp Biol Med 232:454–460, 2007

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Introduction

Cryptosporidium parvum is a protozoan parasite from the *Apicomplexa* phylum, capable of infecting humans by ingestion of oocysts. Once ingested, the parasite anchors the apical membrane of the host enterocytes, leading to a reorganization of the host-cell actin cytoskeleton and the formation of a parasitophorous vacuole with an intracellular but extracytoplasmic location, where the parasite undergoes further development. Although first recognized as an opportunistic pathogen in human, leading to life-threatening diarrhea in immunocompromised patients, *C. parvum* is now regarded as one of the most serious and refractory causes of waterborne-related diarrhea (1, 2). Epidemiologic reports from developing countries have also established the lasting impact of cryptosporidial infection on nutritional status, growth, fitness, and cognitive development in immunocompetent children, thus emphasizing the functional consequences of cryptosporidial infection, particularly when acquired during infancy (3–7). Many antimicrobial drugs have been evaluated to combat *C. parvum* infection; however, none has proved effective in treating the disease. Therefore, much attention should be paid to alleviate the adverse effects of cryptosporidiosis on child development and growth (8).

We previously developed a suckling rat model of acute cryptosporidiosis in which *C. parvum* transiently infests the distal part of the small intestine, with a maximum parasite load on Day 12 and an almost complete clearance of the parasite by Day 16–18 (9). In this model, *C. parvum* is responsible for failure to thrive associated with a dramatic reduction in amino acid absorption at the peak of infection. This malabsorption involves the whole small intestine, whereas only the distal part is parasitized, and is not counterbalanced by any upregulation of amino acid trans-

port, even after spontaneous clearance of the parasite, thus preventing any catch up in growth rate (10).

Absorption of di- and tripeptides in the small intestine occurs mainly through the proton-coupled transporter PepT1 (11). PepT1 is evenly distributed along the small intestine, and this pattern remains similar from Day 4 until adulthood in rats (12). In a preliminary study, we showed that PepT1 mRNA level was maintained in infected intestine and even transiently overexpressed at the peak of infection, in contrast to mRNA coding for amino acid transporters, such as EAAT3 (13). These results suggest a transcriptional upregulation of the peptide transporter in response to parasite development and *C. parvum*–induced malnutrition. However, because the parasite development induces a rearrangement of the brush-border actin network of the enterocyte, post-translational regulation in PepT1 transport activity could not be ruled out. The purpose of the present study was thus to investigate whether or not *C. parvum* infection alters PepT1 expression and activity in our model of acute cryptosporidiosis.

Materials and Methods

Parasites. *C. parvum* oocysts (genotype 2) (gift from M. Naciri, INRA, Nouzilly, France) were initially purified from the feces of a patient infected with human immunodeficiency virus and maintained by regular passage in suckling newborn rats (9). This strain is responsible for a failure to thrive in infected animals (14). The pooled colonic perfusates were kept in 150 m*M* phosphate-buffered saline (PBS; pH 7.2) at 4°C in the presence of penicillin (100 $IU \cdot ml^{-1}$), streptomycin (100 $\mu g \cdot ml^{-1}$), and amphotericin B (0.25 $\mu g \cdot ml^{-1}$) until use.

Experimental Design. A total of 22 litters of specific-pathogen-free (SPF) outbred Sprague-Dawley suckling rats were used in this study: 11 were parasitized with *C. parvum,* and 11 were used as controls. In each group, six litters were used for Western blot analysis, three were dedicated to fluxes measurements, and two were used for γ-interferon (IFN-γ) assays.

Female rats with 4-day-old litters were obtained from Charles River (L'Arbresle, France) and handled according to the regulations of the French Ministry of Agriculture. Food (M20 diet, Special Diets Services, Witham, UK) and water were available *ad libitum*. Each single dam was housed with its litter (adjusted to 10–11 pups) in a separate cage and maintained in an appropriate animal facility. At Day 4, pups were infected by gavage with 100 μl of colonic perfusate containing 5×10^5 cryptosporidia. Rats from the control litters were inoculated by gavage on Day 4 with 100 μl of colonic perfusate obtained from 12-day-old SPF rats free from *C. parvum*.

Animals were killed either on Day 12 (peak of infection) or Day 21 (after spontaneous clearance of the parasite) by intraperitoneal pentobarbital injection. The parasite load of the animals used in each experiment was checked at the time of killing on a 1-cm-long segment of distal ileum, which was homogenized in 10 volume of PBS and then stained with the Ziehl-Neelsen reagent, according to Henriksen's method (15).

***Ex-Vivo* Measurements of Oligopeptides (Glycyl-Sarcosine) Fluxes Across the Ileal Mucosa.** Mucosal to serosal fluxes of glycyl-sarcosine across the intestinal mucosa were determined at Day 12 and Day 21, using ileal sheets mounted in Ussing chambers as previously described (10). Eleven rats originating from different litters were used at each time point in each group. Briefly, a 3-cm segment of ileum, adjacent to the ileo-cecal junction, was rinsed free of intestinal contents, opened along the mesenteric border, and mounted between two halves of a custom-made Ussing chamber with an aperture of 0.39 cm^2 (Marty Technologie, Marcilly sur Eure, France). The mucosal and serosal compartments of the chambers were filled with 3 ml of MES/Tris buffer (pH 6) containing (in m*M*) 25 MES, 140 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$, and 5 glucose. The tissue was oxygenated by a gas lift of O_2/CO_2 (95:5) and the temperature maintained at 37°C throughout the experiment. Transepithelial fluxes of glycyl-sarcosine across the ileal mucosa were measured by adding 100 μ*M* of glycyl-sarcosine (Sigma Chemicals, St. Louis, MO) and 1 μCi of [^{3}H]glycyl-sarcosine (Hartmann Analytics, Braunschweig, Germany) to the MES/Tris buffer in the mucosal compartment of the Ussing chambers. A 20-μl sample was taken from the mucosal compartment 10 min after the addition of radiolabeled glycyl-sarcosine, and 500-μl samples were withdrawn from the serosal compartment every 15 mins for 60 mins and replaced with an equivalent volume of fresh MES/Tris buffer. The amount of radioactivity was measured in the different samples by liquid scintillation counting using a Liquid Scintillation Analyzer 2200 CA (Packard Instrument, Rungis, France) and used to calculate peptide fluxes across the ileal mucosa (expressed as $nmol \cdot cm^{-2} \cdot min^{-1}$) for the 15- to 60-min period.

Western Blot Analysis. *Preparation of Intestinal Epithelial Cells.* Controls and infected rats were sacrificed on Day 12 ($n = 43$ and $n = 34$ for controls and infected rats, respectively) and Day 21 ($n = 6$ for control and infected rats, respectively). Epithelial cells were isolated from intestinal epithelium, as described by Déchelotte *et al.* (16). After opening the abdominal cavity, the whole small intestine was removed and divided into three segments of equal length. The proximal and distal parts were rinsed free of intestinal content with saline, ligatured to one end, filled with 150 m*M* NaCl containing 2.5 m*M* EDTA, ligatured at the other end, and placed in 150 m*M* NaCl for 45 mins at 4°C. Luminal content, containing isolated epithelial cells, was drained, mixed with 4 ml of ice-cold 150 m*M* NaCl, and centrifuged for 3 min at 4°C and 1600 *g*, and the cellular pellet was washed twice with 150 m*M* NaCl. For 12-day-old rats, cell pellets isolates from three rats of the same litter were pooled together to gather a sufficient amount of material. Cell samples were sonicated using a 50-W Vibracell 72434

(Bioblock, Illkirch, France) with 30 pulses, setting 40%, in an ice bath, centrifuged at 9000 *g* for 30 mins at 4°C. The supernatant was added with antiproteases (aprotinin 2 $\mu g \cdot ml^{-1}$, PMSF 1m*M*) and stored at –20°C. Protein concentrations were determined using the bicinchoninic acid kit (Sigma Chemicals) with bovine serum albumin as protein standard.

Preparation of Brush-Border Membrane Vesicles. Controls ($n = 20$) and *C. parvum*–infected rats ($n = 12$) were sacrificed on Day 12. Epithelial cells were isolated from the distal part of the intestine as described before and maintained at 4°C. Samples from three to five rats per litter were pooled and used to prepare brush-border membrane vesicles (BBMV) by the method of Kessler *et al*. (17) but using $MgCl_2$ precipitation instead of $CaCl_2$ to avoid activation of proteases as described by Van Voorhis *et al*. (18). BBMV were added with antiproteases, lysed by sonication for 30 secs in an ice bath, and stored at –20°C before immunoblot analyses. Aliquots were used for protein determination and measurement of sucrase activity.

Sucrase Measurement. Sucrase activity in samples can be considered as representative of brush-border enrichment and cell maturity because it is located in the brush border of epithelial cells in the villi, with an increasing gradient from the crypt-villous junction to villus tip, as previously described for PepT1 (12). This PepT1 gradient was maintained during *C. parvum* infection (13). Briefly, aliquots of epithelial cells samples were incubated at 37°C for 1 hr in the presence of α1-glucopyranosyl-β2-fructofuranoside, the sucrase substrate, and glucose production was measured with glucose oxidase (19).

Immunoblot Analyses. The abundance of PepT1 protein in the isolated epithelial cells and in the BBMV was measured by quantitative Western blot analysis using an affinity-purified rabbit anti-rat PepT1 (1 $mg \cdot ml^{-1}$) produced against the synthetic peptide VGKENPYS-SLEPVSQTNM (Agro Bio, La Ferté St Aubin, France) according to Miyamoto *et al*. (20). Specificity of the rabbit anti-rat PepT1 and the molecular weight of the PepT1 band were controlled by performing immunoblotting with and without preincubation of the anti-rat PepT1 antibody with the synthetic peptide for 1 hr at room temperature.

Proteins from sonicated cell samples or sonicated BBM vesicles (10–25 μg per well) were resolved on a 9% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred on polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 10% nonfat dry milk in TBS-T (20 m*M* Tris-HCl (pH 7.4), 150 m*M* NaCl, 0.05% Tween 20) and probed for 1 hr with the affinity-purified rabbit anti-rat PepT1 (final concentration: 1 $mg \cdot ml^{-1}$). The membranes were washed five times in TBS-T and probed with peroxidase-conjugated goat anti-rabbit IgG 1:10000 (Sigma Chemicals) for 1 hr, both steps at room temperature. After five more washings in TBS-T and TBS (TBS-T without Tween 20), the bound antibodies were detected with an enhanced chemiluminescence immunoblotting system (ECL Plus, Amersham Life Science, Arlington Heights, IL). The intensity of the signal was quantified using a digital imaging system (Alpha Innotech Corporation, San Leandro, CA). PepT1 expression in intestinal epithelial cell was standardized for the amount of protein in the cell lysate. To take into account the differences in brush-border membrane enrichment from one sample to another, sucrase activity was used to normalize the amount of PepT1 in BBMV fractions.

IFN-γ Assay. Control ($n = 6$) and *C. parvum*–infected ($n = 6$) rats originating from different litters were sacrificed on Day 12 and Day 21. Blood was collected by intraperitoneal cardiac puncture then centrifuged at 1600 *g* for 10 mins at 4°C. The serum was stored at –80°C until use. After opening the abdominal cavity, the whole small intestine was removed and divided into three segments of equal length. The proximal and distal parts were rinsed free of intestinal content with 150 m*M* PBS (pH 7.2), homogenized in five volumes of 150 m*M* PBS (pH 7.2) with 2 $\mu g \cdot ml^{-1}$ aprotinin and 1 m*M* PMSF using a glass tissue grinder in an ice bath, and then centrifuged at 9000 *g* for 30 mins at 4°C. The supernatants were stored at –80°C until use. Before carrying out the assay, cells in proximal and distal intestinal homogenates were sonicated using VibraCell 72434 as previously described and then centrifuged at 9000 *g* for 10 mins at 4°C. IFN-γ levels in serum and intestinal homogenates were measured in duplicate by means of a sandwich enzyme-linked immunoabsorbent assay (ELISA) (Rat Interferon-γ ELISA kit, Biosource, Nivelles, Belgium). The sensitivity of the ELISA was <13 $pg \cdot ml^{-1}$ in serum and intestinal homogenate without dilution of samples. Results were expressed as pg of IFNγ per ml for serum and as pg of IFN-γ per mg of total protein for intestinal homogenates.

Statistical Analysis. All data in the figures are expressed as mean ± standard deviation. Statistical analysis of the results was performed by analysis of variance followed by Fisher's protected least significant difference and Student's *t* test for PepT1 and IFN-γ quantification and oligopeptides fluxes analysis, respectively, using the statistical analysis software (Statview, Abacus Concepts Inc., Berkeley, CA). $P < 0.05$ was considered significant.

Results

Oocysts were never detected in controls rats. In *C. parvum*–infected rats, the parasite load was at least 50 oocysts per 50 high-power microscopic field (×400) on Day 12, whereas *C. parvum* oocysts were no longer detected on Day 21.

***Ex-Vivo* Measurements of Glycyl-Sarcosine Fluxes Across the Ileal Mucosa.** The accumulation of glycyl-sarcosine in the serosal compartments of Ussing chambers increased linearly between 15 and 60 mins, in both control and *C. parvum*–infected rats at Day 12 and Day 21. Transepithelial dipeptide fluxes were therefore calculated from the slope of the serosal accumulation during this

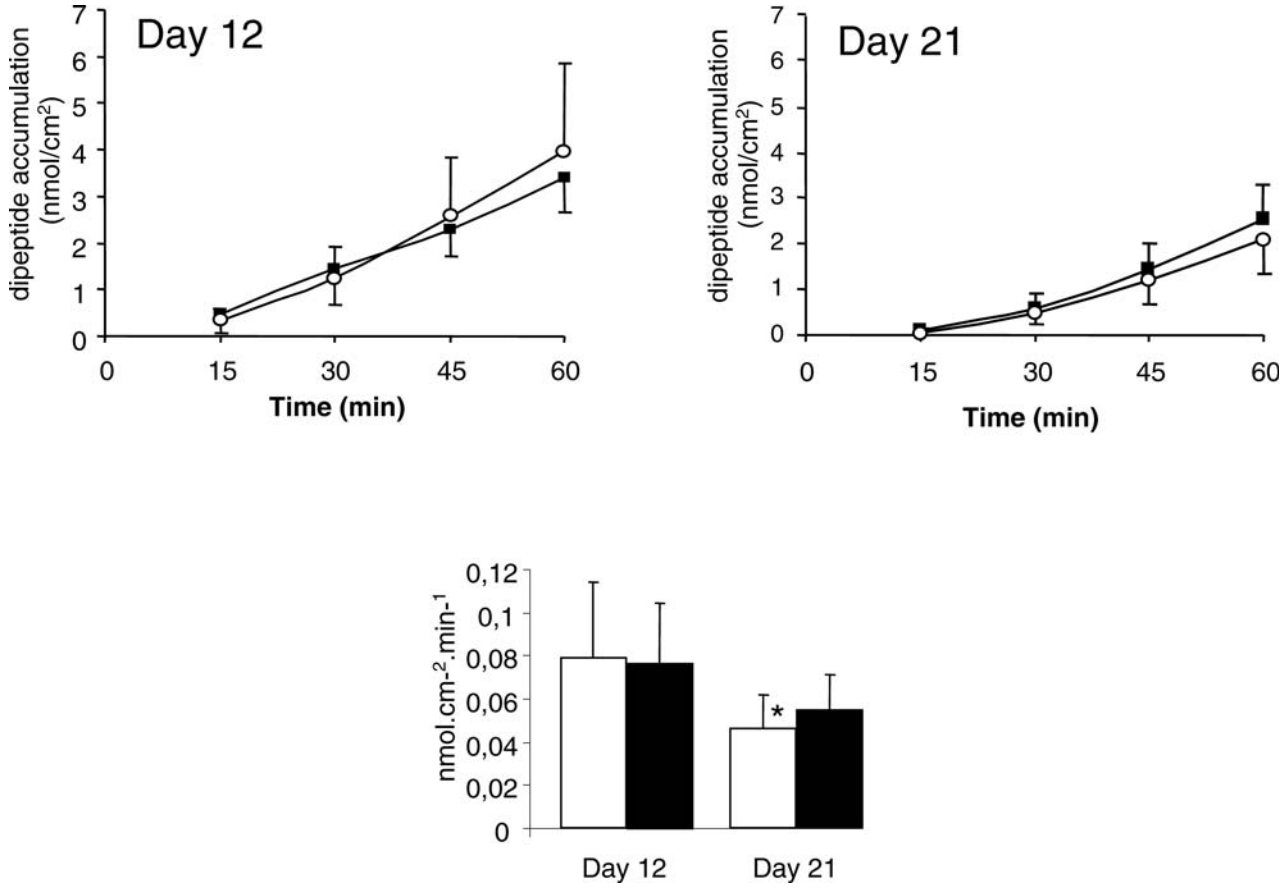

Figure 1. Time course and fluxes of glycyl-sarcosine transfer from the mucosal to the serosal side of *C. parvum*–infected (■, $n = 11$) and control (○, $n = 11$) ileal mucosa at Day 12 and Day 21. In each animal, [^{3}H]glycyl-sarcosine transport per cm^2 was determined over the 15- to 60-min period. The bar graphs represent the fluxes. Results are expressed as mean ± SD. Ileal fluxes were significantly decreased at Day 21 compared with Day 12 in controls ($P < 0.05$) but not in *C. parvum*–infected rats. No significant variation was observed between infected and control tissues at Day 12 or at Day 21.

period. Although lower at Day 21 compared with Day 12 in both control and infected animals, the difference in ileal fluxes of glycyl-sarcosine was significant only in controls ($P < 0.05$). Interestingly, fluxes were similar in *C. parvum*–infected and controls rats, both on Day 12 (0.0760 ± 0.0281 and 0.0784 ± 0.0354 n$M \cdot cm^{-2} \cdot min^{-1}$, respectively) and Day 21 (0.0551 ± 0.0160 and 0.0459 ± 0.0164 n$M \cdot cm^{-2} \cdot min^{-1}$, respectively) (Fig. 1). No interaction between age and infection was observed.

Expression of Total PepT1 Protein in the Isolated Epithelial Cells and Brush-Border Membrane. To further investigate the level of regulation of peptide transport, expression of the PepT1 protein was assessed using Western blot analysis.

As depicted in Figure 2, competitive immunoblotting analysis showed that the affinity-purified rabbit anti-rat PepT1 recognizes a band of 97 kDa, which was specifically blocked by preabsorbing the primary antibody with the immunizing peptide. PepT1 expression in epithelial cell did not vary significantly with age or with the proximo-distal localization in the small intestine and was unaffected by *C. parvum* infection on both Day 12 and Day 21 (Fig. 2).

The subcellular distribution of PepT1 was further investigated at the time peak of infection (Day 12) by measuring its expression in brush-border membranes prepared from isolated epithelial cells. Sucrase activity, which is known to specifically locate in the brush-border membrane, was used to normalize PepT1 expression. There was no difference in sucrase activity between epithelial cells isolated from control and *C. parvum*–infected rats on Day 12 (data not shown).

In contrast to total protein, expression of PepT1 protein in the brush-border membranes was dramatically decreased (–80%, $P < 0.05$) at Day 12 in samples originating from *C. parvum*–infected rats compared with controls (Fig. 2).

IFN-γ Level in Mucosa and Serum. Serum IFN-γ level concentration was increased by 400% at Day 12 in infected compared with control rats (51.7 ± 32.8 pg·ml^{-1} and 9.5 ± 0.5 pg·ml^{-1}, respectively; $P < 0.02$). IFN-γ level in the distal part of the small intestine was 3-fold increased in infected compared with control rats (5.2 ± 1.3 pg·mg^{-1} and 1.8 ± 0.3 pg·mg^{-1} respectively; $P < 0.001$). Although nonsignificant, IFN-γ level was also increased in the proximal part of the small intestine at the peak of infection

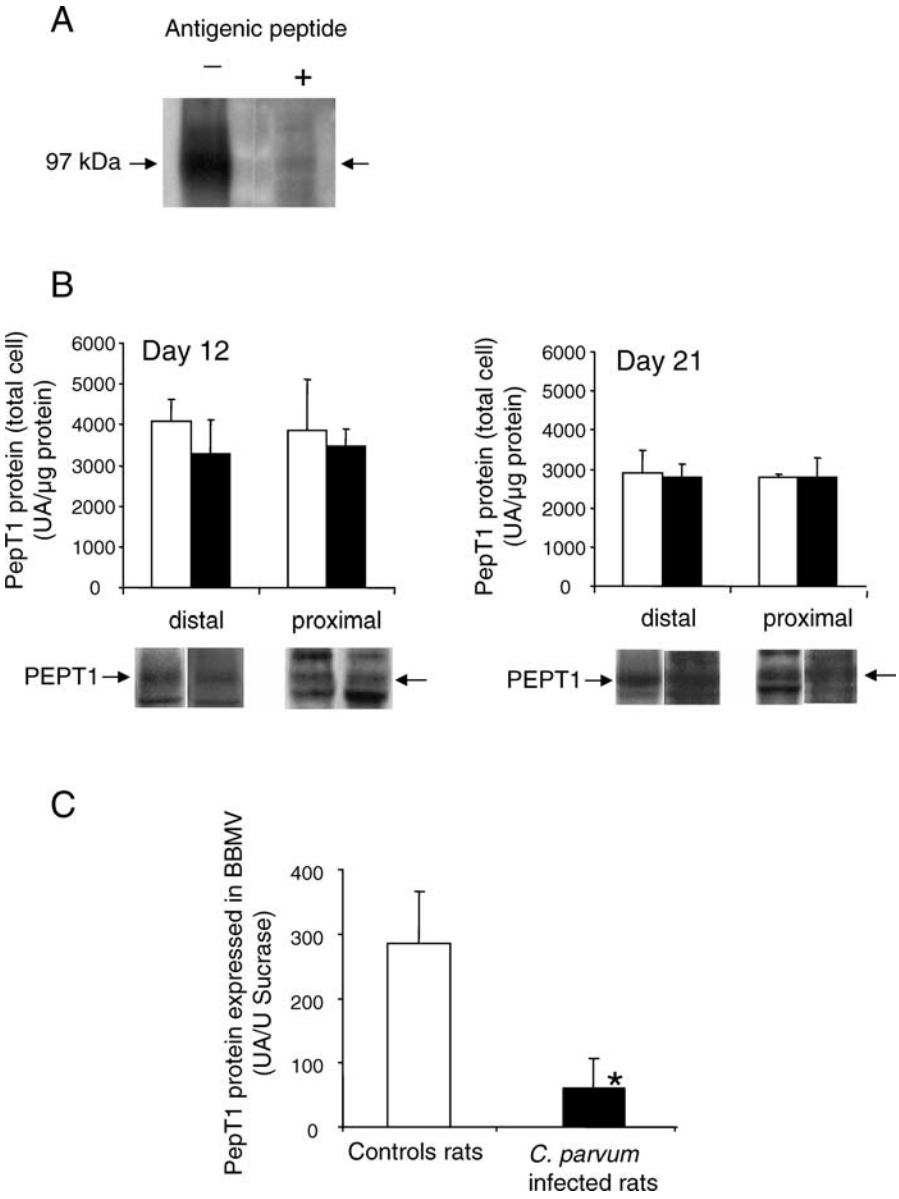

Figure 2. Expression of total PepT1 protein in the isolated epithelial cells and brush-border membrane. (A) Competitive immunoblotting performed with and without preincubation of the affinity-purified rabbit anti-rat PepT1 with the synthetic peptide VGKENPYSSLEPVSQTNM for 1 hr at room temperature. The band for PepT1 is at 97 kDa. (B) Expression of total PepT1 transporter in proximal and distal epithelial cell lysates isolated from *C. parvum*–infected rats (■, $n = 23$ and 6 on Day 12 and Day 21, respectively) and controls (□, $n = 22$ and 6 on Day 12 and Day 21, respectively). For 12-day-old rats, cell isolates from three rats per litter were pooled. Twenty-five (distal) or 20 (proximal) µg of proteins were subjected to 9% SDS-PAGE. Resolved proteins were transferred to PVDF membranes and incubated for 1 hr at room temperature with the anti-PepT1 antibody. Densities of Western blot bands were quantified in regard with total protein of the sample (AU/µg of proteins). (C) Expression of PepT1 protein in BBMV prepared with epithelial cells isolated at Day 12 from distal part of the small intestine in *C. parvum*–infected rats (■, $n = 12$) and controls (□, $n = 20$). Cell isolates from three to four infected rats and from five control animals were pooled. Ten µg of proteins were subjected to 9% SDS-PAGE, transferred to PVDF membranes and then incubated 1 hr at room temperature with the anti-PepT1 antibody. Densities of the bands were quantified in regard with sucrase activity of the sample (AU/U sucrase).

compared with controls (7.0 ± 1.4 pg·mg^{-1} of protein and 4.1 ± 3.5 pg·mg^{-1} of protein, respectively). No more difference was observed at Day 21.

Discussion

We have previously shown that *C. parvum* infection in neonatal rats is responsible for a major amino acid malabsorption, leading to undernutrition, and growth failure that is not counterbalanced by any upregulation, even after spontaneous clearance of the parasite (10, 14). In contrast, the present study shows that peptide transport across the small intestine is unaffected during cryptosporidiosis, despite villous atrophy and a reduced expression of PepT1 in the brush-border membrane, thus suggesting a transient post-translational upregulation of the oligopeptide transport activity. These data suggest a specific role for the di-

tripeptide transporter PepT1 in the metabolic adaptation to cryptosporidiosis-associated malnutrition.

Using the same rat model, we have previously shown that PepT1 mRNA expression was upregulated in the small intestine of *C. parvum*–infected rats at the peak of infection (13). This result agreed with those of other studies reporting an increase in PepT1 transcripts during malnutrition and fast-induced intestinal injury (21–25). However, no increase was observed in total protein expression in our model. This discrepancy should be due to the mucosal secretion of IFN-γ observed during *C. parvum* infection, which has been recently shown to activate proteasome activity (26). Moreover, the present results show no evidence for an upregulation of glycyl-sarcosine transport across the intestinal mucosa of *C. parvum*–infected rats, which remained similar to that of control animals, thus suggesting a more complex regulation of peptide transport in this pathology. Indeed, the results of Western blot experiments show that total PepT1 expression in intestinal epithelial cell was similar in control and infected rats, both in the proximal and distal part of the small intestine, but the amount of PepT1 protein recovered in the brush-border membrane was significantly decreased at the peak of infection in *C. parvum*–infected rats. This suggests that PepT1 targeting to the brush-border membrane is impaired during cryptosporidiosis and that parasite infestation results in transporter segregation within an intracellular pool. This is consistent with the results of a previous immunohistochemical experiment, showing an accumulation of PepT1 immunoreactivity within the enterocyte of *C. parvum*–infected rats at the peak of infection, whereas the staining was restricted to the brush-border membrane in control rats (13). The existence of an intracellular pool of peptide transporters has been reported in Caco-2 cells and rat intestine (27, 28).

Despite the impaired trafficking of peptide transporter to the apical membrane of epithelial cell and the long recognized marked villous and microvillous atrophy responsible for a decrease in intestinal absorption surface, peptide transport across the small intestine of *C. parvum*–infected rats was similar to that measured in control rats. This suggests the existence of additional mechanisms resulting in an increased activity of the transporter during *C. parvum* infection, thus counteracting the consequences of a reduction in the number of transporters available for peptide absorption.

Most attention has been given to the transcriptional regulation of peptide transport in the small intestine (25), and little is known about its post-translational regulation. Buyse *et al.* (29) showed that IFN-γ dose dependently upregulates dipeptide transport through an increase in intracellular pH, thus enhancing the proton electrochemical gradient across the brush-border membrane, which represents the driving force for PepT1. An IFN-γ–dependent regulation of PepT1 was recently confirmed by Vavricka *et al.* (30), who showed that IFN-γ and tumor necrosis factor-α upregulated PepT1 expression and activity in Caco-2 cells. Mucosal IFN-γ is known to play a key role in immune response to *C. parvum* (31–33). In the present study, we showed a 3-fold increase in IFN-γ level in the distal small intestinal mucosa of infected rats at the peak of infection compared with controls. The large increase in IFN-γ may activate the transporter, through the enhancement of the inward proton gradient or alternative mechanisms that remain to be investigated, thus counterbalancing the effect of a reduction in the amount of transporter.

Overall, the present data demonstrate that in contrast to glucose and amino acids, peptide transport across the small intestine is preserved during experimental cryptosporidiosis. The mechanisms responsible for the maintenance of this transport are complex, as illustrated by the increase in the level of transcripts coding for PepT1, the expression of the PepT1 protein at a normal level in the intestinal epithelium, and the decrease in the amount of PepT1 protein recovered in the brush-border membrane. Altogether, these results strongly suggest the involvement of post-translational mechanisms, probably related to the secretion of IFN-γ during this infection.

These results are particularly important for children with cryptosporidiosis and children infected with human immunodeficiency virus, who could benefit from nutritional support to break the vicious circle of malnutrition/immunodepression/cryptosporidiosis.

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