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Molecular & Biochemical Parasitology Release of metabolic enzymes by *Giardia* in response to interaction with intestinal epithelial cells

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Abstract

Giardia lamblia, an important cause of diarrheal disease, resides in the small intestinal lumen in close apposition to epithelial cells. Since the disease mechanisms underlying giardiasis are poorly understood, elucidating the specific interactions of the parasite with the host epithelium is likely to provide clues to understanding the pathogenesis. Here we tested the hypothesis that contact of *Giardia lamblia* with intestinal epithelial cells might lead to release of specific proteins. Using established co-culture models, intestinal ligated loops and a proteomics approach, we identified three *G. lamblia* proteins (arginine deiminase, ornithine carbamoyl transferase and enolase), previously recognized as immunodominant antigens during acute giardiasis. Release was stimulated by cell–cell interactions, since only small amounts of arginine deiminase and enolase were detected in the medium after culturing of *G. lamblia* alone. The secreted *G. lamblia* proteins were localized to the cytoplasm and the inside of the plasma membrane of trophozoites. Furthermore, *in vitro* studies with recombinant arginine deiminase showed that the secreted *Giardia* proteins can disable host innate immune factors such as nitric oxide production. These results indicate that contact of *Giardia* with epithelial cells triggers metabolic enzyme release, which might facilitate effective colonization of the human small intestine.

Keywords

Parasite; Cell–cell interaction; Innate immunity; Secretory product; Arginine deiminase; Enolase

1. Introduction

The intestinal protozoan pathogen *Giardia lamblia* annually infects around 300 million people worldwide [1]. Infection usually starts by ingestion of cysts, which differentiate into

trophozoites that colonize the upper small intestine [2]. Clinical manifestations of giardiasis vary from asymptomatic carriage to chronic diarrhea and severe malabsorption [3]. The mechanisms by which *G. lamblia* causes disease are poorly understood. The parasite is not invasive and little or no mucosal inflammation is seen during acute infection [4]. The severity of disease may depend on multiple parasite and host factors [3,4]. One of the proposed mechanisms is secretion of proteins with toxin-like activities [5,6] but no giardial toxin has been identified to date. *In vitro* interaction models for *G. lamblia* and intestinal cells are well established [7–11], as are rodent models [12–14] and this has increased the knowledge of the infection [15–17].

The overall goal of this study was to identify proteins released by the parasite during its interaction with host cells. Earlier studies had indicated that parasite proteins are released into the growth medium during incubation of axenic cultures of *Giardia* trophozoites in serum-free medium but no specific proteins have been identified so far. However, the highly immuno-reactive variable surface proteins (VSPs) [18,19] and an unidentified 58 kDa protein causing intestinal fluid accumulation in mice [5,20] have been reported to be excretory–secretory factors.

Recently several cysteine-type proteases of *G. lamblia* origin were detected after co-incubations with rat small intestine epithelial cells [15], and other host cell–*Giardia* interaction experiments and giardiasis patient data have shown that *Giardia* reduces the epithelial barrier function [16,17] and induces apoptosis [14,21–23], but the effector protein(s) have never been identified.

To improve our understanding of host–parasite interactions during infection, we asked whether exposure of *G. lamblia* to human intestinal epithelial cells (IEC) might lead to release of trophozoite proteins into the medium. We recently showed that a secreted parasite factor induces chemokine expression in human Caco-2 IECs during *Giardia* interaction [8]. Here we identified three major *Giardia* proteins released into the medium after only brief interaction between *G. lamblia* and IEC. These three enzymes (arginine deiminase (ADI), ornithine carbamoyl transferase (OCT) and enolase) function in giardial metabolism and are immunoreactive during human and murine infections [24,25]. This is the first study identifying proteins in the secretome of *Giardia*, and the first study in a human homologue system (*Giardia* of human origin-human IECs) presenting evidence for interaction related excretory–secretory products of *Giardia lamblia*.

2. Materials and methods

2.1. Reagents and cell culture

Unless otherwise indicated, reagents were obtained from Sigma Chemical Co, USA. *Giardia lamblia* strain WB (ATCC30957), clone C6, and GS, clone H7 (ATCC50581) trophozoites were grown as described [8]. Intestinal epithelial cell lines HT-29, Caco-2, cervical epithelial HeLa cells and human fibroblasts were all grown in high glucose DMEM supplemented with 10% FBS, 4mM L-glutamine, 1 × MEM non-essential amino acids, 160 µg/ml streptomycin and 160 U/ml penicillin G at 37 °C and 5% CO₂. IEC-6 cells were grown according to ATCC specifications. Prior to interaction experiments, the Caco-2 cells were differentiated into small intestine-like enterocytes by post-confluence cultivation for 14–17 days, changing the medium twice weekly. The enterocyte phenotype was confirmed by immunolocalization of ZO-1 and presence of tight junctions and by elevated mRNA expression of intestinal alkaline phosphatase and aminopeptidase N compared to non-differentiated cells [8].

2.2. In vitro host–parasite interaction and protein precipitation

HT-29 or differentiated Caco-2 cells were washed 3 times in 37 °C PBS before initiating interaction with PBS washed trophozoites, with a cell ratio (parasite:IEC) of 3:1. The interacting cells, and controls of IECs and *Giardia* separately, were incubated in culture flasks filled with serum-free M199, supplemented with 6 mM ascorbic acid and 6 mM cysteine, pH adjusted to 7.2, at 37 °C for 2.5 h. The condition of the cells was monitored by phase-contrast light microscopy during the interaction and viable *Giardia* trophozoite numbers were counted before and after interaction using trypan blue staining according to instructions from the manufacturer (Sigma Chemical Co, USA).

After interaction, the culture medium was pre-cleared of cells by centrifugation (2500 rpm, 15 min at 4 °C) and then filtered through a 0.22 µm pore filter (Pall Corporation, USA). Proteins were precipitated over-night at 4 °C with 10% trichloroacetic acid, collected at 2500 rpm, 30 min at 4 °C followed by drying at room temperature. Pellets from interactions and control experiments were dissolved in PBS and equal volumes were analyzed on 2D gels. Samples for 1D Western blot analysis were precipitated similarly, changing the TCA concentration to 20% and adding 0.02% DOC 30 min before the over night precipitation, followed by a wash in cold acetone before drying of the pellet.

2.3. Protein identification by 2D gel electrophoresis and mass spectrometry

Precipitated proteins were separated by 2D gel electrophoresis according to the PS-1 protocol [24], stained and analyzed by mass spectrometry as described [24]. 70- or 180-mm ReadyStrip IPG Strips (BioRad Laboratories Inc., USA), in pI ranges nonlinear 3–10 and linear 5–8, were used for isoelectric focusing. For isolation of proteins for mass spectrometry analysis, gels were stained with Coomassie brilliant blue and all major spots were excised. Protein spots were subjected to in-gel digestion with trypsin (modified sequence grade, porcine; Promega, USA). Peptide spectra were internally calibrated against autolytic peptides from trypsin. Matrix-assisted laser desorption ionization (MALDI) analysis was performed on a Bruker Reflex III mass spectrometer (Bruker-Franzen Analytik, Germany) using alpha-cyano-4-hydroxycinnamic acid as matrix.

Peptide maps were searched against simulated tryptic digests in NCBI database using the Mascot search engine at Matrix science (<http://www.matrixscience.com>). No miss-cleavages were allowed and the peptide mass tolerance was set to <0.2 Da. Significance of identification was evaluated using the Probability Based Mowse Score of the Mascot Program. For confirmation of spot 4 (*G. lamblia* enolase) tandem mass spectrometry was performed on a hybrid quadrupole-orthogonal acceleration time-of-flight (TOF) instrument (Micromass, UK) and the partial amino acid sequence obtained was compared to protein sequences from the *G. lamblia* genome project (www.mbl.edu/giardia).

2.4. Production of recombinant proteins

Enolase, ADI, and OCT were expressed and purified as described [24].

2.5. Polyclonal antibody production

BALB/c mice were immunized intraperitoneally as described [26] on days 0, 15, and 30 with 50 µg of purified recombinant ADI, OCT or enolase re-suspended in 100 µl PBS and emulsified with an equal volume of RIBI adjuvant (Corixa, USA). Prior to initial immunization and after each boost, blood was collected from the tail vein and the serum fraction was assayed for specific antibody titers. The specificity of each polyclonal antibody was determined by Western blot analysis using total *Giardia* extract and the antibodies were shown to be specific.

2.6. Localization of ADI, OCT and enolase in trophozoites

Trophozoites were harvested by chilling on ice for 30 min, washed twice in ice-cold PBS, and fixed with 3% paraformaldehyde for 30 min at room temperature, followed by 5 min in 100 mM glycine in PBS. Fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 30 min and blocked >1 h in 2% bovine serum albumin in PBS. The mouse polyclonal antibodies against ADI, OCT and enolase were diluted 1:200 in PBS containing 0.1% Triton X-100, incubated with fixed cells for 1 h, washed 3 times with PBS and incubated for 1 h with FITC-conjugated sheep anti-mouse antibody (dilution 1:2000, DAKO, Denmark). Fluorescence microscopy was performed on a Leica DM-IRBE microscope using a 100× HCX PL Fluotar lens (Leica Microsystems GmbH, Germany) and digital images were recorded using a cooled CCD camera (Diagnostic Instruments Inc., USA) and processed with the Metaview software package (Visitron Systems GmbH, Germany).

2.7. Localization of proteins during host–parasite interaction

Co-culture of human differentiated Caco-2 cells and *G. lamblia* trophozoites was performed at a 3:1 parasite:host cell ratio for 18 h in 8-well slide chambers. Complete DMEM with serum and antibiotics were used with 5% CO₂ as described [8]. Wells were washed 3 times with warm PBS then fixed and immuno-stained as above. Cell interactions and protein distribution patterns were observed using a Zeiss Axioplan II Imaging fluorescence microscope equipped with appropriate filter sets, an Axiocam CCD camera and AXIOVISION software (Carl Zeiss Light Microscopy, Germany).

2.8. Detection of secreted proteins using Western blot analysis

Secreted proteins from 15 min to 2.5 h co-incubations of confluent HT-29 cells or differentiated Caco-2 cells, HeLa cells, human fibroblasts, IEC-6 rat small intestinal cells and WB-C6 *Giardia* trophozoites, was analyzed by Western blots with antibodies against ADI, enolase and OCT. The secreted proteins were precipitated with 0.02% DOC and 20% TCA followed by a wash in cold acetone for enhanced yield and the resulting pellets were dissolved in 1X SDS Loading Buffer to give a 200-fold final concentration of the released co-culture proteins. For acidic samples, the pH was adjusted to pH 6.8, by addition of NaOH. 10 µl of each precipitation was separated by 10% SDS-PAGE and transferred to a PVDF membrane (Millipore Corporation, USA).

The PVDF membrane was blocked in 5% non-fat dried milk, 1X TBS, 0.1% Tween-20 at 4 °C over-night, incubated with primary antibodies against ADI, enolase or OCT, diluted 1:2000 in blocking solution. Specifically bound primary antibodies were detected by horseradish peroxidase-labeled goat anti-mouse Ig antibody (DAKO, Denmark), diluted 1:30,000 in blocking solution. The membrane was incubated, washed in TBS-Tween and developed using the ECL Plus Western blotting detection system (GE Healthcare, Sweden).

2.9. Identification of proteins secreted in vivo

To examine the secretion of *Giardia* proteins *in vivo*, ligated jejunal loops (2 cm) were prepared in deeply anesthetized adult C57BL/6 mice and infected with 10⁷ trophozoites *G. lamblia* GS/M trophozoites in 200 µl PBS. Control loops were injected with 200 µl PBS alone. Two mice were used per condition. At 4 h the loops were removed, opened up and placed into 1 ml ice-cold intestinal wash buffer (PBS, 1 mM DTT, 2× Complete Protease Inhibitors; Roche). The intestinal washes were first centrifuged (2500 rpm, 15 min at 4 °C) to remove cells, followed by a second centrifugation (13,000 rpm, 15 min at 4 °C) to clear the supernatants from cellular debris. As positive controls the same number of trophozoites were incubated in 1 ml PBS for 4 h at 37 °C, sonicated and centrifuged at 13,000 rpm for 15 min at 4 °C. 200 µl of the supernatant was added to 1 ml intestinal washing buffer from

uninfected control mice to mimic the conditions from the ligated loop experiments. The protein content in each sample was measured by BioRad Bradford analysis, and 5 µg protein was mixed with 6XSDS loading buffer and separated by 10% SDS-PAGE. The ligated loop samples were screened for presence of ADI, OCT and enolase by Western blots as described above. All animal experiments were approved by the UCSD Institutional Animal Care and Use Committee.

2.10. ADI activity and nitric oxide production

Enzymatic activity of purified recombinant ADI was determined after buffer change as described [27]. 100 ng reactive protein or boiled (not shown) in complete DMEM was added to differentiated Caco-2 epithelial cells in 12 well-plates (Corning, USA) containing 1 ml cell culture medium per well. The cells were stimulated for nitric oxide (NO) production by addition of IL-1 (20 ng/well), IFNα (50 ng/well) and TNF (20 ng/well) simultaneously with ADI or buffer control. At 18 h post cytokine addition, growth medium was collected and analyzed for total nitrite production using the Total Nitric Oxide kit (Promega, USA). Quantification of total nitrate of un-stimulated Caco-2 cells and stimulated Caco-2 with only protein buffer added was used as controls.

3. Results

3.1. Identification of secreted proteins by 2D gel electrophoresis and mass spectrometry

Giardia trophozoites were cultured *in vitro* with or without confluent intestinal epithelial cells (differentiated Caco-2 or HT-29) in serum-free medium for up to 2.5 h and monitored by light microscopy. The trophozoites were motile and maintained normal morphology throughout the experimental period. Live trophozoite numbers, determined by trypan blue staining, did not differ significantly before and after interaction, suggesting absence of major cell lysis (data not shown). After 3 h of co-incubation in a low oxygen environment (closed caps, filled 250 ml culture flasks) the intestinal epithelial cells began to swell and detach from the surface of the flask. This was not seen if epithelial cells were cultivated in co-cultures with serum and an oxygen-rich environment (in open 6-well plates).

Samples of culture media from 2.5 h interactions between *Giardia* and human intestinal cells were analyzed by 2D gel electrophoresis and silver staining of the gels (Fig. 1A–C). The same dominant proteins were detected on 2D gels with interaction media of both HT-29 (Fig. 1A) or differentiated Caco-2 cells (Fig. 1C) and *Giardia* cells. The dominant protein spots were much less abundant when parasites were incubated without intestinal cells (Fig. 1B). All visible spots, equivalent to the most dominant spots of silver stained gels (Fig. 1A–C), were excised from a Coomassie stained 2D gel, digested in-gel with trypsin and analyzed by mass spectrometry.

Peptide mass fingerprints were compared to theoretical digests of proteins from all species at NCBI. *G. lamblia* OCT and ADI were identified in the co-culture supernatant. Tandem mass spectrometry analysis was performed for final identification of spot number 4, which revealed *Giardia* enolase (Table 1). In addition, human alpha-enolase (syn. enolase-1) and heat shock 70 kDa protein 8 (HSPA8) were identified. HSPA8 is too highly conserved to distinguish between human and bovine origin, but it was only detected in the medium of co-incubated cells indicating specific release by the human cells during the interaction. A constant background of bovine serum proteins was identified in the medium of co-cultures and in the controls, and several spots of lower intensity, possibly corresponding to interaction related proteins, failed to be identified (Fig. 1A–C). The 2D pattern of soluble protein fractions of *Giardia* trophozoites is much more complex than that of the co-culture supernatants (data not shown, [24]) supporting the idea that the results are not due to large scale lysis of trophozoites. For example, the abundant cytoplasmic, metabolic enzymes

fructose-1,6-biphosphate aldolase and UPL-1 [24] were not detected after co-incubation (Fig. 1).

3.2. Localization of ADI, OCT and enolase in *Giardia* trophozoites

The released *Giardia* proteins OCT, ADI and enolase identified here have important metabolic roles in the parasite [28], but have not previously been localized. We produced recombinant versions of the three proteins and raised polyclonal antibodies in mice. Each serum reacted with one band of the expected molecular weight in Western blots (data not shown). Immunofluorescence microscopy showed that ADI and Enolase exhibited a punctuate pattern within the cytoplasm (Fig. 2A and C), while OCT localized in the cytoplasm with highest accumulation prominently near the plasma membrane (Fig. 2B). During co-incubation with intestinal cells the cytoplasm was stained with all antibodies but there were also differences in the staining pattern from separately grown parasites; ADI localized to the most anterior part of the parasite (Fig. 2G) and OCT and enolase appeared to be more concentrated in the central anterior area (Fig. 2H and I).

3.3. Western blot analysis of ADI, OCT and enolase release in vitro and in vivo

To further analyze protein release during host–parasite interactions, we used the specific polyclonal antibodies against OCT, ADI and enolase in Western blots of supernatants precipitated from interactions between trophozoites and human IECs. Fig. 3A shows that ADI and enolase, and to a lesser degree OCT, were secreted into the growth medium within 15 min of interaction. Co-cultivation of *Giardia* and differentiated Caco-2 cells for 15 min gave a 7-fold higher secretion of ADI and a 3-fold higher enolase secretion compared to *Giardia* grown without epithelial cells (Fig. 3A). Incubation of *Giardia* trophozoites with HT-29, human cervical epithelial cells (HeLa), human fibroblasts and rat small intestinal epithelial cells (IEC-6) also induced release of the enzymes (Fig. 3B). Note however that the level of released ADI relative to enolase is different compared to the differentiated Caco-2 cell experiment (Fig. 3A and B). The difference in relative amounts of released ADI and enolase between HT-29 and differentiated Caco-2 cells is also obvious in the 2D analysis (Fig. 1A and C, spots 3 and 4). These experiments show that there is no specificity for a certain cell-type but the relative levels of secreted ADI was higher after incubation with the differentiated human intestinal epithelial cells (Caco-2) compared to the other cell lines. It should also be noted that *Giardia* trophozoites only interact with intestinal epithelial cells during a natural infection, which can explain the relaxed cell type specificity for protein release.

The *in vivo* release of *Giardia* enzymes was studied in C57BL/6 mice infected with *G. lamblia* GS trophozoites, the only human *Giardia* strain colonizing mouse small intestine. Fig. 4 shows that enolase and a fragment of ADI (indicated by an asterisk) are detected in the intestinal luminal fluid from *Giardia*-infected ligated loops whilst OCT could not be detected in these samples. These data are consistent with the observation that giardial ADI and enolase are released during host–*Giardia* interaction.

3.4. Characterization of the function of released *Giardia* proteins

Enolase and ADI are released both *in vitro* and *in vivo* during host–*Giardia* interactions. Enolase has been shown to be a multi-functional enzyme often secreted by pathogens at mucosal surfaces [29]. However, the function of secreted enolase is poorly understood and we were not able to show any specific extra-cellular activity of enolase.

The released *Giardia* protein with the relatively greatest increase upon interaction of *Giardia* and differentiated human intestinal epithelial cells was ADI (Fig. 3A and B). This enzyme plays an important role in the intracellular arginine metabolism of *Giardia* [28]. We showed

earlier that *Giardia* deplete arginine required by intestinal epithelial cells for nitric oxide (NO) production [19]. Secreting ADI, may enable the parasite to disarm the potentially detrimental effects of NO-dependent host defenses. To test this, we evaluated the effect of recombinant *Giardia* ADI on epithelial cell NO-production. The differentiated, small intestine-like epithelial cell line Caco-2 was stimulated for nitric oxide production by the addition of cytokines (IL-1 α , IFN- γ and TNF- α), together with enzymatically active recombinant *G. lamblia* ADI. As seen in Fig. 5, active, but not boiled (data not shown) *Giardia* ADI suppressed the production of NO, as evidenced by decreased levels of the stable NO end product, nitrite, in the growth medium. Thus, ADI may directly interfere with the mucosal immune system and thus facilitate *Giardia* infection in the small intestine.

4. Discussion

The pathogenesis of intestinal infections with *Giardia lamblia* remains elusive [4]. This protozoan parasite is not invasive and no conventional toxin has been identified [1]. Any mechanism that supports its ability to remain in the host small intestine or to resist host defenses may be viewed as a virulence factor. Here we present data showing release of three *Giardia* enzymes, previously only characterized as being involved in the intracellular energy metabolism of *Giardia* [28].

Several previous studies have searched for secreted proteins correlated to disease and colonization by *G. lamblia*. The VSPs that cover the entire trophozoite surface [30], were originally identified as spontaneous excretory–secretory proteins [31,32]. A 58 kDa non-VSP protein, reported to localize to the surface of *G. lamblia* P-1 strain [5], was found in the culture supernatant of trophozoites incubated in serum-free medium [5,20]. However, the published partial amino-acid sequence from the 58 kDa protein (ADFVPQVST) does not resemble any sequence in the *Giardia* WB genome (data not shown), and it only partially fits with the contaminating protein bovine serum albumin (BSA). It is noteworthy that we have seen that full-length BSA and several different sized proteolytic fragments of BSA bind to the trophozoite surface strongly and are slowly released during incubation in serum-free medium (data not shown). It is possible that these fragments interfered with the identification of the 58 kDa protein. Moreover, several unknown proteins with masses ranging from 15 to 225 kDa as well as un-identified proteins with cysteine-type protease activity have been reported in supernatants of *Giardia*-host cell co-cultures [15,33,34].

This is the first time giardial ADI, OCT and enolase have been shown to be present in *Giardia* culture supernatants. These typically cytoplasmic proteins have been found to be surface-exposed or secreted in other organisms. We were not able to detect any signal sequences in the proteins but now there are many examples of secreted proteins in eukaryotes without signal sequences [35]. Enolase has been implicated in the pathogenesis of other microbes colonizing mucosal surfaces [29,36]; prokaryotic *Streptococcus agalactiae*, *S. sobrinus*, *S. pyogenes* and eukaryotic *Candida albicans* α -enolases are secreted [36,37], while other enolases are surface-associated [29,38,39]. Some of these enolases are immunodominant [29,36], as is *Giardia* enolase [24,25]. However, we were not able to show any specific activity of secreted *Giardia* enolase and its direct role, if any, during host–parasite interactions remains to be determined.

In addition to enolase we have demonstrated host-induced secretion of OCT and more extensively ADI, two *Giardia* enzymes of the arginine metabolic pathway. This is an unusual, bacterial-like pathway, not present in human host cells. Trophozoites use ADI and OCT to actively metabolize arginine for energy, thus depleting arginine from the growth medium. Arginine depletion is known to induce apoptosis in human cell lines [40] and human giardiasis patients show an increased rate of apoptosis of intestinal epithelial cells

[23]. This has been suggested to be a major disease mechanism [22]. Further experiments will show if ADI is involved in this induction of apoptosis. Prior to inducing apoptosis, this depletion reduces the ability of IECs to produce NO, an anti-microbial innate defense molecule. Our previous studies implicate NO toxicity to *Giardia* because *in vitro* NO donors inhibit giardial growth but not viability [19]. NO also inhibits both encystation and excystation of *Giardia* and could thus interfere with parasite transmission [19]. Many intracellular pathogens are killed by NO, but the role of NO in controlling infections of extracellular pathogens is not well established [19]. Interestingly, the NO levels in intestinal epithelial cells have also been shown to be important in the regulation of adsorption/secretion of water [41], suggesting that it could be associated with symptoms of giardiasis. The secreted OCT and ADI of *Giardia* might reduce the levels of intestinal arginine further and lower the NO production by IECs. In support of this hypothesis, recombinant mycoplasmal ADI reduced NO production in human cells [18,42]. When we studied the effect of recombinant giardial ADI on IEC *in vitro* we also observed a decrease of NO production from the intestinal epithelial cells. Secretion of arginine metabolizing enzymes could thus be a general mechanism used by pathogenic microbes at mucosal surfaces. Therefore, arginine consumption and NO reduction define a novel cross talk between *Giardia* as a non-invasive pathogen and the host intestinal epithelium.

Our present and earlier results confirm that *Giardia lamblia* is involved in an extensive cross talk with the host intestinal epithelial cells. Immunomodulating proteins produced by microbes are important virulence factors since they are crucial for the survival of the microorganisms in the host. Our results show host-cell stimulated secretion of giardial proteins, which have the potential to be involved in the immunological events during initiation of infection and subsequent survival of *Giardia* in the host. We have also recently shown that the host response towards *Giardia* of differentiated Caco-2 cells is partly mediated by soluble factor(s) [8]. The secreted proteins we identify here are immunodominant in human infections [29,36] and experimental mouse infections [13]. The targeting of secreted giardial proteins for immunoneutralization may thus represent a useful therapeutic strategy, aimed at treating or preventing *Giardia* infections. Further experiments will show what roles these proteins may play during *Giardia*-host cell interactions.

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Abbreviations

IEC	intestinal epithelial cell
ADI	arginine deiminase
OCT	ornithine carbamoyl transferase
NO	nitric oxide

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Fig. 1. Precipitated culture supernatant proteins from 2.5 h incubations of *Giardia lamblia* trophozoites with (A) and without human HT-29 cells (B) and with differentiated small intestine-like Caco-2 cells (C). Identified *Giardia* and human proteins are indicated by numbers 1–5 and the identities of the proteins are described in Table 1. Encircled spots were identified as contaminating full-length protein or fragments of bovine serum albumin. Unmarked spots failed to be identified. The figure shows representative silver stained 2D electrophoresis gels. However, identification was performed from Coomassie stained gels.

Fig. 2. Localization of identified proteins in *G. lamblia* trophozoites by polyclonal mouse antibodies (green) raised against purified recombinant ADI (A), OCT (B) and enolase (C). Corresponding light microscopy pictures are shown in panels (D–F). Figs. (G–I) shows the localization of ADI (G), OCT (H) and enolase (I) during interaction with differentiated Caco-2 cells.

Fig. 3.

Detection of secreted *Giardia* proteins during human cell–trophozoite interactions *in vitro*. The presence of ADI, enolase and OCT was detected in concentrated culture supernatants from co-cultures of *Giardia lamblia* WB-C6 and differentiated Caco-2 human intestinal epithelial cells and to lesser extent *G. lamblia* trophozoites alone (A). The ADI secretion increases 7-fold within the first 15 min upon interaction, while enolase increases 3-fold. In (B) released ADI, enolase and OCT levels are compared between co-cultures of HT-29, HeLa cells, human fibroblasts and IEC-6 rat small intestinal epithelial cells. To yield visible bands on the Western blot 2.5 times more supernatant proteins from the IEC-6 co-culture was loaded on the gel compared to the other co-culture supernatant precipitates.

Fig. 4.

Detection of secreted *Giardia* proteins during host cell–trophozoite interaction *in vivo*. ADI (A) and enolase (B) were detected by Western blot analysis of intestinal washes after 4 h *Giardia* GS-H7 infection of C57BL/6 mice ligated intestinal loops. Arrows indicate sizes for full-length ADI (A) and enolase (B). The asterisk in panel A, lane 3, is a proteolytic fragment of ADI, also seen in sonicated *Giardia* trophozoites in lane 1. All blots show a strong background from the secondary antibody. Lanes 1: sonicated *Giardia* GS-H7 trophozoites; Lanes 2: Intestinal wash from PBS mock infected mice; Lanes 3: intestinal wash from *Giardia* GS-H7 infected mice. Lane 4 (panel A) was loaded as lanes 3, but only subjected to the secondary antibody.

Fig. 5. Secreted nitric oxide levels measured by colorimetric substrate conversion of total nitrate in the growth medium of differentiated Caco-2 epithelial cells after stimulation with cytokines (+) in the absence or presence of recombinant *G. lamblia* ADI. The bars represent the mean $\pm$ S.D. of three values per treatment.

Table 1
Identified proteins from 2D gels of interaction supernatant of *G. lamblia* and HT-29 co-cultures

Spot	MW (kDa) ^a	pI ^a	Sequence coverage ^b	Expect value ^c	Sequenced ^d	Identity (accession no.)
1	37	6.8	43%	3.5E-12		Giardia OCT (AAC28939)
2	52	7.1	25%	7.7E-0.3		Human α-Enolase (P06733)
3	66	6.2	26%	7.1E-0.8		Giardia ADI (AAC06116)
4	48	5.6	14%	0.2	(1346,76) LGPQEYMIAPTK	Giardia α-Enolase (AAL73509)
5	69	4.1	25%	8.5E-06		HSP70, protein 8 (P11142)/(P19120) ^e

^aData from 2D gel.
^bMatching masses from MALDI-TOF mass spectrometry when searching against all entries in NCBI using Mascot search engine.
^cProbability value obtained from Mascot search.
^dSequence obtained by MS/MS analysis. Parental monoisotopic mass of sequenced peptide.
^eHuman and bovine HSP70 are too conserved to be distinguished.