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Original article

Elucidating pathways of *Toxoplasma gondii* invasion in the gastrointestinal tract: involvement of the tight junction protein occludin

Caroline M. Weight [a,c,1,2], Emily J. Jones [a,c,2], Nikki Horn [a], Nikolaus Wellner [b], Simon R. Carding [a,c,*]

[a] *Gut Health and Food Safety Institute Strategic Programme, Norwich Research Park, Norwich, NR4 7UA, UK*
[b] *Analytical Sciences Unit, Institute of Food Research, Norwich Research Park, Norwich, NR4 7UA, UK*
[c] *Norwich Medical School, University of East Anglia, Norwich Research Park, Norwich, NR4 7UA, UK*

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Abstract

Toxoplasma gondii is an obligate intracellular parasite infecting one third of the world's population. The small intestine is the parasite's primary route of infection, although the pathway of epithelium transmigration remains unclear. Using an *in vitro* invasion assay and live imaging we showed that *T. gondii* (RH) tachyzoites infect and transmigrate between adjacent intestinal epithelial cells in polarized monolayers without altering barrier integrity, despite eliciting the production of specific inflammatory mediators and chemokines. During invasion, *T. gondii* co-localized with occludin. Reducing the levels of endogenous cellular occludin with specific small interfering RNAs significantly reduced the ability of *T. gondii* to penetrate between and infect epithelial cells. Furthermore, an *in vitro* invasion and binding assays using recombinant occludin fragments established the capacity of the parasite to bind occludin and in particular to the extracellular loops of the protein. These findings provide evidence for occludin playing a role in the invasion of *T. gondii* in small intestinal epithelial cells.

Keywords: *Toxoplasma gondii*; Occludin; Invasion; Intestinal epithelial cells

1. Introduction

The ability of *Toxoplasma gondii* to infect almost any warm blooded animal and virtually any nucleated cell makes it the most prevalent parasitic infection worldwide. It is estimated that up to one third the world's human population is infected, although prevalence varies between countries [1,2]. In the United States, it is estimated that approximately 22% of the population 12 years and older have been infected with *T. gondii* whereas in certain South American countries, the frequency of seropositive individuals is as high as 75% [3]. With the exception of the immunocompromised and pregnant women, *T. gondii* causes a relatively asymptomatic infection of typical fever-like symptoms. The majority of infections occur following the consumption of contaminated, undercooked meat, unwashed vegetables and contaminated water supplies [4,5]. The gastrointestinal tract is therefore a major route of *T. gondii* infection in most cases [6,7]. Tachyzoites are the life form of *T. gondii* that disseminate out of the gut and migrate through the body and infect the brain and muscles, where they convert to bradyzoites that form dormant, long lived and non-immunogenic cysts [8]. How the parasite transmigrates intestinal epithelial cells is unclear, although there is evidence that the paracellular pathway is important for parasite dissemination [9].

* Corresponding author. Institute of Food Research, Norwich Research Park, Norwich, NR4 7UA, UK. Tel.: +44 (0) 1603 251410.
E-mail address: Simon.Carding@IFR.ac.uk (S.R. Carding).
[1] Present address: Immunology Section, Leukocyte Migration Group, Department of Experimental Medicine, BMC D14, Lund University, Lund, Sweden.
[2] Authors contributed equally.

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The small intestinal epithelial barrier consists of a single layer of intestinal epithelial cells (IECs) that separate the luminal contents from the underlying mucosa. These cells express apico-lateral junctional proteins, the most apical of which is the tight junction (TJ). TJs provide a barrier for the regulated passage of ions, uncharged molecules and macromolecules. They consist of a complex of over 100 proteins, the interactions of which determine barrier function. Prominent TJ proteins include the claudin family members that control permeability, junctional adhesion molecules that govern cell polarity and migration, and the MARVEL proteins such as occludin, which regulates permeability to macromolecules, while a variety of other integral membrane proteins, peripheral membrane proteins and signaling proteins such as Zonula occludens-1 (ZO-1) make up the remaining TJ complex [10–12]. TJs are dynamic in nature and often consist of mobile pools within the membrane and cytoplasm that are involved in recycling and turnover of the protein. In the case of occludin this mobility is associated with changes in phosphorylation status [13,14].

TJs are targeted by pathogens as a mechanism of host invasion. For example, the enteric pathogens *Vibrio cholera* and *Clostridium perfringes* secrete proteases and enterotoxins, respectively, that degrade occludin and claudins [15]. A paracellular route of entry between cells via intercellular adhesion molecule 1 (ICAM-1) by *T. gondii* has been reported [9] and we have previously shown that *T. gondii* bradyzoites and cysts affect the cellular distribution of occludin in barrier epithelial cells both *in vitro* and *in vivo* [16,17].

Using epithelial cells derived from within the crypts of Lieberkühn of the murine small intestinal epithelium, we investigated the pathways by which *T. gondii* invades (defined as infection into cells and transmigration between cells via the paracellular pathway) the intestinal epithelium.

2. Materials and methods

2.1. Cells

The rodent small IEC lines m-IC_{cl2} and IEC-6 were maintained as previously described [18,19]. To reduce occludin expression, m-IC_{c12} cells were cultured either on 13 mm coverslips (for H&E staining), in 6 well plates (for immunoblotting), or on transwell cell culture inserts (for transmigration assays). In each case 0.38 μg of occludin-specific siRNA (a mixture of three 19–25 nucleotides, Santa Cruz) in transfection media (OptiMEM, Invitrogen) was added to the cell cultures for 6 h at 37 °C, washed and then incubated for a further 24 h in normal growth media. As a control, m-IC_{c12} cells were incubated with scrambled (non-silencing, scRNA) siRNAs (Santa Cruz). Occludin knockdown was assessed by immunoblotting and immunocytochemistry. Bead arrays (30 Plex Bead Mixture, BD Biosciences) were used to quantify cytokines and chemokines in cell supernatants, according to the manufacturers' instructions and analyzed using a Cytomics FC500 MPL (Beckman Coulter).

2.2. Parasites

The type 1 RH strain of *T. gondii* tachyzoites stably expressing YFP [20] were maintained by continuous passage in confluent monolayers of Hs27 Human Fetal Foreskin Fibroblasts (European Collection of Cell Cultures) in DMEM supplemented with 2 mmol/L L-Glutamine and 10% FBS at 37C in 5% CO_2. Pelleted parasites were collected after 90% HFF lysis by centrifugation at 1000 g for 15 min.

2.3. Transmigration and infection assays

m-IC_{cl2} cells were plated onto the apical compartment of polyethylene terephthalate (PET) cell culture transwell inserts (8 μm pore size, BD Biosciences) within a 24 well plate. TEER was measured using an Epithelial Tissue Volt Ohmmeter 2 (World Precision Instruments). By day 13, inserts contained confluent, polarized monolayers of cells. Barrier permeability was assessed by periodic TEER measurements and flux of FITC-conjugated dextran (3–5 kDa; Sigma–Aldrich) across the transwell membrane; 1 mg/ml FITC-dextran was added to the apical compartment and media from the basal compartment was analyzed for FITC content using a FLUOstar OPTIMA microplate reader (BMG Labtech). FITC-dextran quantification was determined from a standard curve generated using standards of known concentration. Transmigrating parasites were identified from the basal compartment by centrifugation and analyzing by flow cytometry using a Cytomics FC500 MPL. Data was analyzed post-collection using FlowJo version 7.6 (TreeStar).

2.4. Immunocytochemistry

m-IC_{c12} cells were fixed in either 2% formaldehyde (to visualize the parasites) or acetone (to visualize the TJ proteins), permeabilized with 0.2% Triton X-100 and incubated with blocking buffer (0.2% Triton X-100, 3% BSA, 3% goat serum, 3% fish skin gelatin in PBS) prior to incubation with primary antibodies including occludin, claudin-2 (Invitrogen), ZO-1 (Santa Cruz) and β-catenin (BD Biosciences). Controls consisted of either no primary antibody or isotype matched antibodies of irrelevant specificity. A 1:1 mixture of Rhodamine-peanut and -wheat germ agglutinin (Vector Labs) was used to visualize the apical membrane. For transwell cultures, the PET membrane was extracted from the insert and placed cell side up onto a glass microscope slide with DePeX (BDH) and covered with a glass coverslip. To visualize intracellular parasites, m-IC_{c12} cells grown on 13 mm diameter glass coverslips (BDH), fixed (2% formaldehyde), permeabilized and H&E counterstained before mounting and viewing using an upright or inverted LSM510 META on a Zeiss AxioVert 200M microscope. Images were analyzed on LSM software or AxioVision image viewer. Z stacks were composed of 1 μm interval sections with the 40× objective unless stated otherwise. To visualize occludin by Z stack, cells were marked for the apical and basal membrane using surface carbohydrates and β-catenin respectively. This provided a