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Giardia duodenalis Assemblage-Specific Induction of Apoptosis and Tight Junction Disruption in Human Intestinal Epithelial Cells: Effects of Mixed Infections

Wan Hon Koh, Thomas Geurden*, Tim Paget†, Ryan O'Handley‡, Robert F. Steuart§, R. C. Andrew Thompson, and Andre G. Buret||, School of Veterinary and Biomedical Sciences, Murdoch University, Western Australia 6150; *Laboratory of Parasitology, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium; †Dept. of Chemistry, Davis Hall, Lehman College-CUNY, 250 Bedford Park Blvd. West, Bronx, New York 10468; ‡School of Animal and Veterinary Sciences, The University of Adelaide, 5005, Australia; §School of Biomedical Sciences, Faculty of Health Sciences, Curtin University, Perth W.A. 6845, Australia; ||Biological Sciences, Inflammation Research Network, University of Calgary, Calgary T2N 1N4, Canada. Correspondence should be sent to: *aburet@ucalgary.ca*

ABSTRACT: In view of the interest in genotype-specific pathogenesis in *Giardia duodenalis*, the aim of the present study was to examine the effects of infection with different, or mixed, *G. duodenalis* assemblages on the integrity of human intestinal epithelia. To that end, human epithelial cells (HCT-8) were cultured and exposed to different *G. duodenalis* assemblages (A, B, and E) or a combination of these assemblages. Epithelial disruption and apoptosis were evaluated by fluorescent microscopy and apoptotic oligonucleosome quantification. The results indicate that infection with trophozoites disrupts epithelial tight junctions and induces varying degrees of enterocyte apoptosis, depending on the infecting assemblage. All disruptions were caspase-3 dependent and were more pronounced when caused by a non-host specific assemblage. Furthermore, infections by isolates in combination with isolates from another assemblage enhanced the epithelial disruption and apoptosis. Further studies in vitro and in vivo are required to confirm the mechanisms of enhanced pathogenicity of mixed or non-host specific (or both) *G. duodenalis* infections. Findings in the present study point to the potential pathogenic importance of intra-species polyparasitism in giardiasis.

Giardia duodenalis (syn. *Giardia intestinalis*, *Giardia lamblia*) is a major cause of enteric disease in humans, domestic animals, and wildlife worldwide. An estimated 280 million cases of human giardiasis are reported annually, and the infection was recently added to the WHO's "Neglected Disease Initiative" (Thompson and Monis, 2004; Savioli et al., 2006). The pathophysiology of diarrhea in giardiasis is due at least in part to malabsorption of electrolytes, nutrients, and water, maldigestion, and hypersecretion (Buret et al., 1992; Chin et al., 2002; Scott et al., 2004; Buret, 2007, 2008; Panaro et al., 2007; Troeger et al., 2007; Cotton et al., 2011).

The basic mechanisms of pathophysiology in giardiasis remain incompletely understood. Observations from both in vitro and in vivo models demonstrate that the colonization of the small intestine by *Giardia* sp. increases epithelial permeability (Hardin et al., 1997; Scott et al., 2002). Loss of barrier function during *Giardia* sp. infection results at least in part from disruptions in tight junctional proteins including zonula occludens-1 (ZO-1), claudin-1, α-actinin, and cellular F-actin (Teoh et al., 2000; Scott et al., 2002; Harhaj and Antonetti, 2004; Musch et al., 2006; Troeger et al., 2007). Tight junctional disruptions occur prior to shortening of microvilli and disaccharidase deficiencies (Scott et al., 2002, 2004). Thus, the disruption of tight junctional ZO-1 is considered an early marker of pathophysiology in giardiasis (Buret et al., 2002; Chin et al., 2002). The subsequent increase in epithelial permeability is caused by enterocyte apoptosis in a caspase-3 dependent manner (Chin et al., 2002; Panaro et al., 2007; Troeger et al., 2007). Micro-array analyses further demonstrated that exposure of intestinal epithelial cells to *Giardia* sp. trophozoites modulates cell apoptosis (Roxstrom-Lindquist et al., 2005).

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The clinical symptoms associated with giardiasis include acute to chronic diarrhea, abdominal cramps, bloating, nausea, weight loss, dehydration, and decreased appetite. In developing countries, *Giardia* sp. infections have been associated with failure to thrive and reduced cognitive function (Thompson, 2001; Thompson and Monis, 2004; Buret and Cotton, 2011). The mechanisms responsible for the broad spectrum of symptoms caused by *Giardia* sp. infections remain obscure. Part of this variation can be attributed to epidemiological factors, such as infection pressure, or to host factors such as immune status, nutritional status, and age but may also relate to the variable genetics of the parasite (Buret and Cotton, 2011). To date, *G. duodenalis* has been reported to exist in 8 distinct genetic "assemblages" (A to H). Assemblages A and B can infect human hosts, and both isolates are capable of causing symptomatic diarrheal disease (Thompson and Monis, 2004; Robertson et al., 2010). These 2 assemblages also exhibit phenotypic heterogeneity including variation in mean generation time, growth rates, and drug sensitivity (Thompson, 2001; Thompson and Monis, 2004). Putative enterotoxins in excretory–secretory products of *Giardia* sp. have yet to be directly implicated in pathogenesis (Kaur et al., 2001; Shant et al., 2004).

Several epidemiological studies on human giardiasis have attempted to correlate the infecting *G. duodenalis* assemblage with clinical outcome, although with conflicting results (Homan and Mank, 2001; Thompson, 2001; Read et al., 2002; Haque et al., 2005; Sahagún et al., 2008; Breathnach et al., 2010; Robertson et al., 2010). Previous studies in vitro suggested an isolate-dependent induction of enterocyte apoptosis (Cevallos et al., 1995; Chin et al., 2002), although these studies mainly investigated assemblage A isolates. The aims of the present study were to assess and compare the effects of distinct human *G. duodenalis* assemblages, and importantly their combination, on the integrity and apoptosis of human epithelial cells.

Epithelial monolayers were infected with either a single *G. duodenalis* assemblage or a combination of different *G. duodenalis* assemblages (see below). Several parameters were monitored in order to examine potential differences in virulence between assemblages. All experiments were performed using the human ileocecal adenocarcinoma cell line HCT-8 (ATCC CCT-244), cultured in modified "Roswell Park Memorial Institute" (RPMI) medium (Invitrogen, Carlsbad, California) supplemented with 10% heat-inactivated fetal bovine serum (FBS) as validated previously (Chin et al., 2002; Panaro et al., 2007). Briefly, the cells were incubated in 25-cm^3 tissue culture flasks at 37 C with 5% CO_2 in 96% humidity. Once reaching 90% confluency, the cells were trypsinized and 400 µl of suspended cells (1.0×10^5/ml) were then transferred into 8-well Lab-Tek™ chamber slides or 48-well microtiter plates (Nalge Nunc International, Naperville, Illinois) containing serum-free RPMI. The chamber slides were incubated, again until 90% confluency, and a suspension of 1×10^6 trophozoites in 400 µl of RPMI (without serum, "infected") or vehicle RPMI ("control") was added to each test well (final volume 2.5 ml). Three infected replicates and 1 negative control were performed for each assemblage or combination of assemblages simultaneously, and the entire study was repeated 3 times for each parameter measurement. For the mixed infections (A/B, B/E, and A/E), 5×10^5

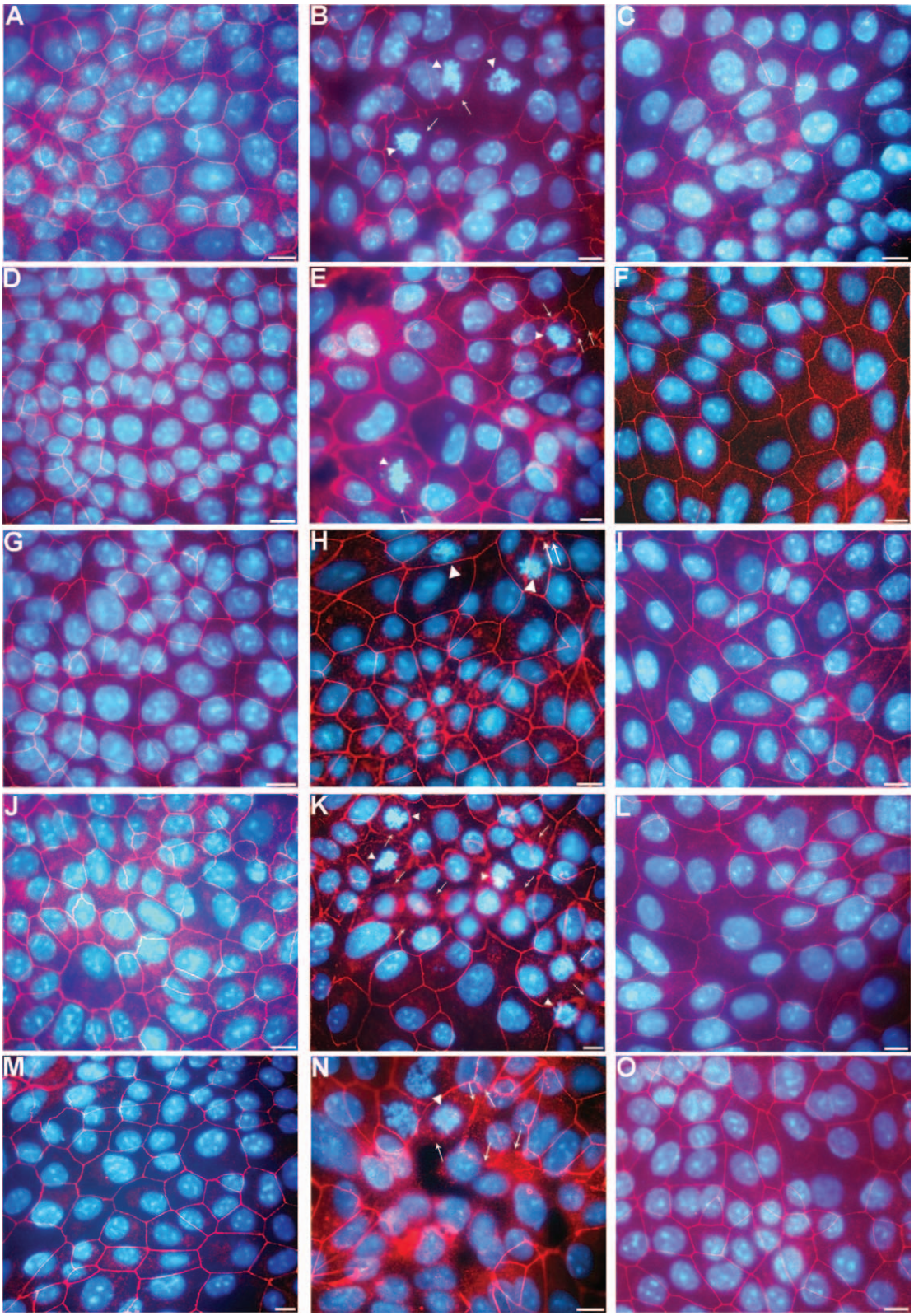

FIGURE 1. Epithelial tight junctional ZO-1 (in red) and cell nuclei (in blue) of human intestinal cell monolayers exposed to *G. duodenalis* (row 1: assemblage A; row 2: assemblage B; row 3: assemblage BAH12c14; row 4: assemblage E; row 5: assemblage A + B). Arrowheads indicate nuclei with condensed chromatin, a marker of cell apoptosis. Arrows indicate disruptions of tight junctional ZO-1, either as interruptions along the cell-cell margins or as abnormal cytosolic accumulations. The first column of panels A, D, G, J, and M are representative micrographs of preparations before addition of *Giardia* isolates. The middle column of panels B, E, H, K, and N are representative micrographs of monolayers co-incubated with *Giardia*. The last

trophozoites of each assemblage in RPMI, making an equivalent total of 1×10^6 trophozoites, were transferred into the test wells.

Axenized trophozoite clones representing the human assemblages A and B, and the livestock assemblage E, were used for the experiments. The isolates used were BAH2c16 (assemblage A), BAH 34c8 (assemblage B), BAH12c14 (a clone with a genotype containing mixed assemblage A and B traits), and P15c1 (assemblage E). The assemblage characterization was determined using the 18S rDNA gene as recently described (Covacin et al., 2011). The sub-assemblage was determined using tpi and gdh genes as previously reported (Lasek-Nesselquist et al., 2009). BAH2c16 was confirmed to be assemblage A, sub-genotype A1. BAH34c8 was confirmed to be assemblage B with a sub-genotype difficult to ascertain in view of its heterogeneity. Finally, BAH12c14 was confirmed to have traits of both assemblage A2 sub-gentoype as well as assemblage B. The isolates used in this study have been characterized previously (Koudela et al., 1991; Meloni et al., 1995). Trophozoites were grown in modified TYS-I-33 medium, containing 10% (v/v) newborn calf serum, in 10-ml flat-bottomed Nunclon tubes (Nunc, Rochester, New York). Trophozoite growth was monitored on a daily basis using an inverted microscope (Olympus BXK 51; Olympus, Tokyo, Japan). The isolates were subcultured every 48 hr or until they reached confluence to maintain the *Giardia* sp. clone. For large-scale trophozoite production, 1-L Schott bottles filled with 10 ml borosilicate glass culture tubes were used to increase the adhesion surface for the trophozoites.

When harvesting the trophozoites, the medium containing dead trophozoites was removed while the culture was still warm. Afterwards, sterile phosphate buffered saline (PBS) (4 C) with penicillin and streptomycin (10,000 unit penicillin and 10 mg streptomycin/ml) (Sigma, St. Louis, Missouri) was added to the culture, and the tube was placed on ice for 30 min to detach the trophozoites by cold shock and centrifuged at 3,500 rpm (270 *g*) for 10 min at room temperature. Supernatants were removed, centrifuged again, and the pellet was washed twice more with sterile PBS supplemented with penicillin and streptomycin (10,000 unit penicillin and 10 mg streptomycin/ml). The washed pellet containing the trophozoites was then resuspended in PBS or RPMI medium, as needed. For infection, the trophozoites were enumerated using a hemocytometer, and a dilution in RPMI was made to the desired number of trophozoites per milliliter (see above).

To test the effects of different assemblages on the tight junctional ZO-1 protein, 8-well chamber slides were incubated with *Giardia* sp. trophozoites at 37 C and 5% CO_2 in 96% humidity for 24 hr, as this was previously shown to be a reliable and biologically relevant time point for observations on the pathogenic effect of *Giardia* sp. on enterocytes in vitro (Chin et al., 2002). Mono-infections with assemblage A, B, and E and the BAH12c14 clone (as listed above), as well as mixed infections with assemblage A/B and A/E, were performed. After incubation, the medium and unattached trophozoites were aspirated from the chamber slides, and each well was washed twice with PBS (4 C) for 5 min at room temperature to detach *Giardia* sp. trophozoites and remove debris. The HCT-8 monolayers were then fixed with 400 µl of ice-cold methanol for 20 min at 4 C. Chamber slides were washed 3 times with sterile PBS, and FBS was added at room temperature for 15 min to block non-specific antibody binding. After 2 washings with cold sterile PBS (10 min), monolayers were incubated with 100 µl of rabbit anti-ZO-1 antibody (1:100 in PBS containing 2% FBS, Zymed Laboratories, San Francisco, California) for 1 hr at 37 C in a dark, humid chamber. Monolayers were washed with cold sterile PBS and incubated for 1 hr at 37 C in a dark, humid chamber with 100 µl of Alexa 555-conjugated goat anti-rabbit secondary antibody (1:2,000 in PBS containing 2% v/v FBS, Invitrogen). Each slide was washed twice with cold PBS prior to HOECHST fluorescent nuclear staining (1 µM) (Molecular Probes, Eugene, Oregon) for 10 min at room temperature. Nuclear staining was used as a marker of cell apoptosis, as validated previously (Chin et al., 2002). After washing with cold PBS, monolayers were mounted using Aqua Poly Mount (Polyscience, Warrington, Pennsylvania). Tight-junctional ZO-1 integrity and cell apoptosis were examined using an optical fluorescent photomicroscope (Model BX 51; Olympus). Each experiment, including all experimental groups, was repeated 3 times. Five fluorescence micrographs were taken from the center of each well on each chamber slide.

The effect of *Giardia* sp. on the induction of apoptosis was further examined in a second series of experiments by inhibiting pro-apoptotic caspase-3. The caspase-3 inhibitor II Z-Asp(OCH_3)-Glu(OCH_3)-Val-Asp(OCH_3)-FMK (Z-DEVS-FMK; Calbiochem, La Jolla, California) is known to inhibit apoptosis irreversibly. Prior to infection with *Giardia* sp. trophozoites, HCT-8 monolayers in chamber slides were treated with the caspase-3 inhibitor for 15 min at 37 C or with vehicle (control), as previously described (Chin et al., 2002). Monolayers were washed with 37 C sterile PBS and the integrity of tight junctional ZO-1, and the degree of cell apoptosis, was assessed as above. This approach has been previously validated, and this caspase-inhibitor does not alter parasite viability or attachment (Chin et al., 2002).

In another set of experiments, the induction of cell apoptosis was quantified using cell death ELISA as previously described (Chin et al., 2002). For these experiments, 10^5 trypsinized cells were transferred into a 48-well plate (Lab-Tek, Nalge Nunc). The culture medium was replenished daily until the monolayer reached 90% confluence. Each well was then infected with 10^6 *Giardia* sp. trophozoites as described above or given the control vehicle. Mono-infections with assemblage A, B, and E and the BAH12c14 clone, as well as mixed infections with assemblage A/B, B/E, and A/E, were performed. Pro-apoptotic topo-isomerase-I (2 µg/ml) (Sigma) was used as a positive control for apoptosis. Apoptosis was measured in duplicate for individual and mixed assemblages after 24 hr based on previous studies indicating that this time point reliably identified the early event of epithelial pathology upon exposure to *Giardia* sp. (Buret et al., 2002; Chin et al., 2002; Scott et al., 2002). The Cell Death Detection Elisa Plus (Roche Applied Science, Indianapolis, Indiana) was used following the manufacturer's instructions. This quantitative sandwich immunoassay specifically measures the histone region of mono- and oligonucleosomes that are released during apoptosis and is a quantitative approach to measure apoptosis. The photometric reaction was measured using an MRX-TC Revelation microplate reader (Magellan Biosciences, Chelmsford, Massachusetts) at 405 nm after 25 min.

Where appropriate, results were expressed as mean ± standard deviation of the mean. A Kruskal–Wallis, followed by a Dunn's test, was performed to examine statistical differences between all the isolates. A Mann–Whitney *U*-test was used for paired comparison between different isolates. *P*-values less than 0.05 were considered to be of statistical significance.

The effect of infection with different assemblages of *G. duodenalis* on the confluent HCT-8 epithelial cells was visualized after 24 hr by comparison of ZO-1 disruption and apoptotic nuclear degradation in the epithelial monolayers. As illustrated in Figure 1, experiments assessed the effects of BAH2c16 (assemblage A; panels A, B, and C), BAH34C8 (assemblage B; panels D, E, and F), BAH12c14 (assemblage A/B; panels G, H, and I), and P15c1 (assemblage E; panels J, K, and L) as test isolates (Fig. 1). Live trophozoites were found in similar numbers at the 24-hr time

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column of panels C, F, I, L, and O are representative micrographs of monolayers treated with a caspase inhibitor prior to co-incubation with *Giardia*. Assemblages A or B, used alone, only caused minor epithelial alterations (B and E). Assemblage A/B (H) caused overt epithelial disruptions, as did assemblage E (K) and the combination of A + B (N). Pre-treatment with a caspase inhibitor totally abolished the *Giardia*-induced tight junctional abnormalities as well as the increase in apoptotic nuclei (C, F, I, L, O). The figures shown are representative of 3 separate experiments, each of which comparatively assessed 5 areas per group each time.

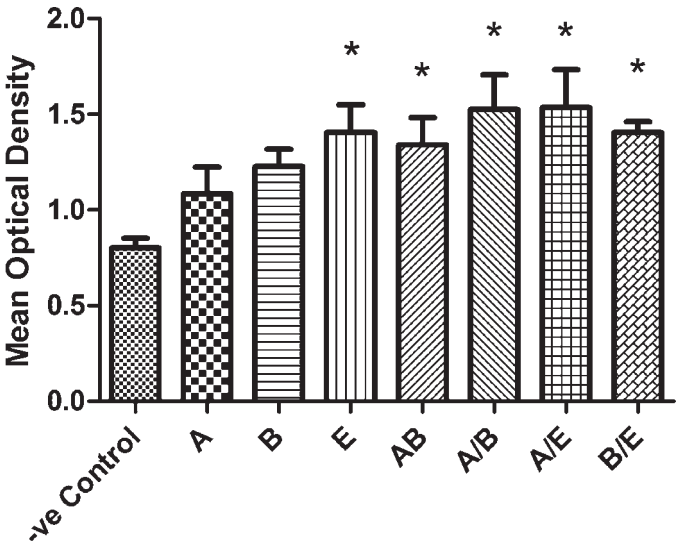

FIGURE 2. Apoptosis levels after infection of the HCT-8 epithelial cells during 24 hr with different isolates, either as a single infection (assemblage A, B, A/B isolate BAH12c14, or E) or as a mixed infection (combination of assemblage A/B, A/E and B/E), as measured by the Cell Death Detection ELISA Plus. In the present experimental setting, isolates BAH12c14 and assemblage E, but not assemblages A or B alone, significantly increased epithelial apoptosis. All assemblages, when used in mixed combinations, significantly elevated enterocyte apoptosis. n = 8 per group. $*P < 0.05$ versus control monolayers.

point and in all preparations. Control epithelia exhibited characteristic per-cellular ZO-1 staining. In contrast, exposure to *Giardia* sp. disrupted tight junctional ZO-1 in an assemblage-specific manner. Abnormalities included focal disruption and punctuate relocalization of ZO-1 along the pericellular junction and in the cytosolic compartment. The effects of the mixed assemblage A/B isolate (BAH 12c14) were much more pronounced than those of assemblage A clone BAH2c16 (which caused no overt disruption) or assemblage B clone BAH34C8 (which caused few disruptions), both of which only had minor effects on their own. In contrast, the same number of trophozoites obtained from a mixed culture of assemblage A clone BAH2c16 and assemblage B clone BAH34C8 dramatically disrupted tight junctional ZO-1 and nuclear integrity (Fig. 1, panels M, N, and O). Infection with the non-human assemblage E also disrupted ZO-1 and fragmented cell nuclei (Fig. 1, panels J, K, and L). Disruptions of ZO-1, in each instance, were associated with nuclear fragmentation (Fig. 1). In an attempt to establish a cause-to-effect relationship between these assemblage-specific effects, ZO-1 disruption was assessed in the presence of a caspase-inhibitor. ZO-1 disruptions were consistently abolished by pre-treatment with the caspase-3 inhibitor (Fig. 1; panels C, F, I, L, and O). The caspase-3 inhibitor also prevented *Giardia*-induced nuclear fragmentation (Fig. 1; panels C, F, I, L, and O).

The caspase-3 inhibition experiments reported here indicated a pivotal role for enterocyte apoptosis in the pathogenesis of tight junctional disruption. Therefore, the pro-apopotic effects of the *G. duodenalis* preparations were assessed with a cell death ELISA in an attempt to assess the isolate-dependent virulence further (Fig. 2). Assemblages A or B on their own failed to significantly increase apoptosis compared to controls. In contrast, the BAH12c14 clone, with A and B genotypic characteristics, as well as assemblage E, significantly induced epithelial apoptosis when administered alone. When assemblages A and B were mixed (as well as with A/E and B/E mixtures), enterocyte apoptosis was significantly elevated versus controls.

The aim of the present study was to examine *G. duodenalis* assemblage-specific effects on human intestinal epithelial integrity and to investigate the effects of mixed infections on epithelia. Moreover, the present report describes findings using 4 *Giardia* sp. isolates that had not been tested in this manner previously and for which the assemblage they belong to has been identified. Consistent with previous observations (Buret et al., 2002; Chin et al., 2002; Scott et al., 2002; Panaro et al., 2007), the results indicate that infection with *G. duodenalis* induces human enterocyte apoptosis as well as disruptions of tight junctional ZO-1 in a caspase-3-dependent manner. Previous studies reported that different *G. duodenalis* isolates have different pathological effects in a neonatal rat model or in epithelial cells in vitro (Cevallos et al., 1995; Chin et al., 2002; Scott et al., 2002) than is seen with isolates from unknown, or the same, assemblages (mostly A assemblages). Here, we demonstrate that these effects are not only isolate specific but also assemblage specific. Indeed, most severe abnormalities after infection with single isolates were observed in epithelia exposed to BAH12c14, a clone with mixed assemblage A and B genotypic characteristics, or in those epithelial monolayers exposed to assemblage E, a genotype believed to infect livestock but not humans (Monis et al., 2009; Jerlström-Hultqvist et al., 2010). Interestingly, the mono-infection with assemblage E (known to infect livestock) induced more-severe ZO-1 disruptions and apoptosis in the human HCT-8 epithelia than did mono-infections with representatives of human-specific assemblages A or B. Additional studies are now required to confirm the mechanisms of tight junctional disruptions in giardiasis, including more-quantifiable approaches as well as assessments of effects on proteins other than ZO-1. Similarly, the significance of these observations in the context of clinical disease warrants further investigation, certainly by using other possible *Giardia* sp. strain combinations but also in the context of host–parasite co-evolution and adaptation (Thompson and Lymbery, 1996).

Most interestingly, findings from the present study demonstrate for the first time that infection with mixed isolates from assemblage A and B together may elicit significantly more epithelial disruption and enterocyte apoptosis than will a mono-infection with the same isolates. Little is known about mixed infections, also referred to as "poly-parasitism," in the context of enteric protozoan infections, either at the intra- or the inter-specific levels. Apart from leading to competitive interactions, poly-parasitism may lead to stimulation of different components of the immune system that may act synergistically (Koukounari et al., 2010). Other studies suggest that this, in turn, may alter the pathogenic behavior of 1, or more, of the co-habiting infectious agents; this may have an additive or synergistic (or both) impact on nutrition and pathogenicity (Pullan and Brooker, 2008). Intriguingly, the isolate BAH12c14 also proved pathogenic as a mono-infection when compared to mono-infections with well-characterized assemblage A or B isolates. Although initially typed as assemblage B, this BAH12c14 isolate, obtained from a remote Aboriginal community in the north of Western Australia where mixed infections are known to occur in some individuals, was later found to contain genetic characteristics of both assemblages A and B (Hopkins et al., 1999; Lasek-Nesselquist et al., 2009). Regardless, the combination of both assemblages A and B, either when expressed in the same isolate or when delivered by mixed infections of 2 different isolates, seems to elicit significantly increased pathogenicity.

How a mixed infection may elicit a more pronounced pathology than mono-infections has yet to be explained. Further studies are needed to assess whether, in an environment with restricted access to nutrients, a single assemblage may face the possibility of extinction in the presence of a competing assemblage (Tibayrenc and Ayala, 1987), laying the basis for attempts at outcompeting the other (Thompson and Lymbery, 1996). Further research in vitro and in vivo is warranted to determine whether, in a competitive environment, these isolates might survive and cause a greater amount of pathology than might others. It would also be most interesting now to determine how mixing different assemblages may affect pathogenicity on epithelial cells by looking at parasite attachment, secretory–excretory products, intra-parasitic virulence regulation, or even modulation of the host response during combined infections.

Together, the data presented here may point to novel cross-talk mechanisms between different isolates belonging to the same pathogen species. These mechanisms may shed new light on the broad spectrum of

clinical outcomes in giardiasis. Recent reports indicate that, on average, 12% (Sprong et al., 2009), but occasionally up to 32.4% (Geurden et al., 2009), of human infections are by mixed templates. A large part of these mixed templates are probably mixed infections, further underscoring the significance of the present findings. The observation that the non-human–specific assemblage E results in greater pathology compared to the human assemblages A and B might also be of relevance, despite the fact that this assemblage is, at present, not frequently reported in human patients. In a recent study, dog and livestock-specific assemblages were described in chinchillas when appropriate molecular tools were used, whereas only assemblage B was found when the classic PCR-sequencing approach was used (Levecke et al., 2011). In addition, assemblage B has been found to be highly prevalent in cattle, next to assemblage A and E (Dixon et al., 2011). These recent studies suggest that hosts may be infected more frequently than expected with other than the host-specific assemblages and that mixed infections may not necessarily be detected with classical approaches of assemblage identification (Meloni et al., 1988; Mahbubani et al., 1992; Hopkins et al., 1999; Covacin et al., 2011). Further investigations need to analyze whether isolates with mixed assemblage characteristics, or combined infections with more than 1 isolate (or both), may indeed elicit greater pathology in vivo and, thus, contribute to the broad spectrum of clinical outcomes reported in giardiasis.

In giardiasis, parasite genotype alone cannot fully explain why infected individuals experience such a broad range of symptoms or lengths of infection. Indeed, recent studies have reported that either assemblages A or B may cause diarrheal disease and that the same assemblage may elicit differing lengths of symptomatic infection in healthy individuals (Hanevik et al., 2005; Robertson et al., 2010; Cotton et al., 2011). Moreover, while various reports from around the world have attempted to correlate parasite genotype with symptomatology even though some have suggested that assemblage B may cause more-severe disease, the observations remain largely conflicting (Homan and Mank, 2001; Thompson, 2001; Read et al., 2002; Robertson et al., 2010; Buret and Cotton, 2011). Whether the greater genetic variability found in assemblage B compared to assemblage A may in part lead to higher pathogenicity requires investigation (Thompson, 2001; Read et al., 2002; Sprong et al., 2009). Regardless, the present findings shed new light on the potential role of mixed infections in the pathogenesis of giardiasis. More research is warranted to test this novel and intriguing hypothesis.

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