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Adaptive immune response in symptomatic and asymptomatic enteric protozoal infection: evidence for a determining role of parasite genetic heterogeneity in host immunity to human giardiasis

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Abstract

The genetic basis of the ultimate clinical outcomes of human giardiasis has been the subject of numerous investigations. We previously demonstrated roles for both host and parasite factors in determining the outcome of enteric infection in a murine model of *Giardia duodenalis* infection. In the current study, fecal and serum specimens from healthy controls and human subjects infected with the intestinal parasite *G. duodenalis* were assessed. Using a semi-nested PCR method, clinical isolates were genetically characterized based on the *gdh* and *tpi* loci, and the phylogenetic trees were constructed. Using a sandwich ELISA method, the serum levels of representative T_{H1} and T_{H2} cytokines were measured in infected human subjects and healthy controls. Here we showed that symptomatic human giardiasis was characterized by significantly elevated serum levels of the T_{H1} cytokine IFN- γ compared to healthy controls, whereas asymptomatic human subjects and healthy controls had comparable levels of serum IFN- γ . Further analyses showed that

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Conflict of interest

The authors have no conflict of interest to declare.

human subjects infected with *G. duodenalis* genotype AI had significantly elevated levels of serum IFN- γ and IL-10, but not IL-5, whereas human subjects infected with AII had similar levels of those cytokines compared to healthy controls. These data demonstrate roles for both host and parasite factors in the determination of the outcome of enteric infections and may further broaden our understanding of host-parasite interaction during enteric protozoal infections.

Keywords

human; protozoa; *Giardia duodenalis*; cytokines

1. Introduction

Human giardiasis is one of the most prevalent enteric parasitic protozoan infections globally, with prevalence rates ranging from 2-5% in the developed world and 20-30% in the developing countries [1]. The causative agent, *Giardia duodenalis* (syn. *G. lamblia*, *G. intestinalis*), is a flagellated protozoan parasite which infects a broad range of mammalian hosts [2, 3]. Human infections are initiated by the ingestion of the quadrinucleated cysts with contaminated food and water. Symptomatic infections in humans are characterized by a wide range of gastrointestinal symptoms that may include abdominal cramps, flatulence, diarrhea, nausea, malnutrition and/or stunted growth, especially in children [4, 5]. The clinical outcomes of human giardiasis vary considerably, with most infections being considered sub-clinical and self-limiting, although re-infection in endemic areas also ensue [1].

Several lines of evidence suggest that the immune response to the human parasite *G. duodenalis* relies on both innate and adaptive arms of the immune system. Although host defense mechanisms required for the control of *Giardia* infections are not fully understood, several reports have shown the requirement of intact innate and adaptive immune mechanisms to control giardiasis in humans and animals [6]. In this regard, previous studies have demonstrated the induction of T_{H1} (IFN- γ , TNF- α), T_{H2} (IL-4, IL-10, IL-13) and T_{H17} (IL-17A) cytokines following infection with *G. duodenalis* or *G. muris* in murine models of *Giardia* infection [7-9]. Consistently, the critical roles of T_{H1} cytokines have been demonstrated in host resistance to giardiasis; mice deficient in TNF- α [10] or IL-6 [11-13] had an impaired ability to control infection in a murine model of *G. duodenalis*. Moreover, the potential necessity of T_{H1} cytokines in host defense against this parasite has been reinforced by elevated levels of these cytokines in the peripheral blood of human subjects infected with *G. duodenalis* [14, 15]. Alternatively, increased levels of T_{H2} cytokines (i.e., IL-5, IL-13) were also observed in human subjects infected with *G. duodenalis* [15, 16] but remarkably mice lacking either IFN- γ or IL-4 were able to control infections with kinetics analogous to their wild-type (WT) littermates [17].

G. duodenalis consists of at least eight distinct lineages or cryptic species called “assemblages”, of which only two lineages (i.e. assemblages A and B) have been known to infect humans [18]. Several contradictory studies have reported that genetically-diverse *Giardia* isolates differ in their propensity to induce immunopathology, and that some isolates

are associated with symptomatic disease, whereas others are not [3, 8]. By way of example, the vast majority of parasites isolated from children with asymptomatic giardiasis belonged to assemblage B (90%), whereas only 3 and 7% of cases were detected to harbor assemblage A or had mixed infections, respectively [19]. Conversely, another study showed that assemblage B was associated with symptomatic disease in the human subjects, whereas only 20 and 6% of human subjects were infected with assemblage A or had mixed infections, correspondingly [20]. Others found no significant associations between the genetic characterizations of the parasite and symptomatic disease [21].

Different mechanisms have been suggested as to the varied clinical outcomes of *Giardia* infections [3, 8]. However, it has been proposed that genetically-different isolates of the parasite induce immune responses in an assemblage- or genotype-specific manner. Alternatively, it also has been proposed that the variations seen in the tendency of genetically-different isolates to induce symptomatic disease are associated with the presence or absence of a given virulence factor [22].

It has already been shown that both host and parasite factors contribute to immunopathology following infection with *G. duodenalis* [8]. The host proinflammatory cytokines (e.g. IFN- γ) contribute to the immunopathology associated with a wide variety of microbial infections [8, 23]. The parasite-induced immunopathology during giardiasis, as determined by the impairment of intestinal disaccharidases, was CD8⁺ T cell-dependent [24], whereas SCID mice and mice deficient in CD8⁺ T cells ($\beta 2M^{-/-}$) did not exhibit any symptoms of disaccharidase deficiency [8]. Furthermore, the immunopathology induced during giardiasis in a murine model of *G. duodenalis* was assemblage-dependent, and mice infected with the GS/M-83-H7 strain (assemblage B), but not the WB clone-C6 strain (assemblage A) of *G. duodenalis*, differed in the absence or the presence of induced disaccharidase deficiency in host [8].

Here we hypothesized that the cytokine profiles in human subjects with symptomatic human giardiasis would differ from human subjects with no overt clinical symptoms, and that human subjects infected with genetically-diverse *G. duodenalis* isolates would differentially secrete cytokines in response to the pathogen. In the present study we investigated the serum cytokine levels of human subjects with symptomatic human giardiasis and asymptomatic cyst passers as well as the association of a given *G. duodenalis* genotype with elevated levels of IFN- γ , IL-5 and IL-10. This study is the first to report immune response profiles in human subjects infected with genetically-distinct *G. duodenalis*. An insight into the multilayer network of host-pathogen interactions during human infection with *G. duodenalis* should assist us to identify novel therapeutic targets for the intervention of this cosmopolitan infection.

2. Materials and Methods

2.1. Study subjects

Fecal and serum specimens included in this study were collected from healthy controls ($n = 38$) and human subjects infected with *G. duodenalis* ($n = 40$) from Kerman, central Iran. Human giardiasis is frequently reported from this area, and it has been reported that up to

47% of residents in some areas are infected with this parasite [25]. Subjects were defined as individual with a positive stool microscopy for the presence of trophozoites/cysts of the parasite, confirmed by a subsequent stool antigen detection test for *G. duodenalis*. Subjects co-infected with other intestinal parasites, as determined by three consecutive stool microscopy using a Ritchie's formalin-ether concentration method were excluded from the analysis. The healthy controls were recruited from individuals negative for *G. duodenalis* using both stool microscopy and a stool antigen detection test. Stool antigen detection-based assays are considered reliable and reproducible for the diagnosis of infections with *G. duodenalis* and have been reported to correlate excellently with PCR-based methods [26, 27].

2.2. Ethics statement

Written informed consents were obtained from infected human subjects and healthy controls that participated in this study or their parents/legal guardians in case of children. This study was approved by the Ethical Committee of Kerman University of Medical Sciences and the Iranian Ministry of Health, Treatment and Medical Training Protection Code of Human Subjects in Medical Research.

2.3. Fecal consistency scoring

Stool consistency and stool scores were defined according to a stool consistency scoring system described elsewhere [28, 29], where scores of 1 to 4 indicated well-formed, soft, loose, and watery stool consistency, respectively.

2.4. Parasitological procedures

Trophozoites and/or cysts of *G. duodenalis* in fecal specimens were identified by light microscopy on wet mounts stained with iodine solution. In brief, fecal pellets from infected human subjects were washed once with ice-cold PBS (pH 7.4), and resuspended in ice-cold PBS. Using a sucrose gradient centrifugation technique, fecal suspensions were subsequently layered on 0.85 M sucrose and centrifuged at 600×g for 10 min at 4°C to further purify cysts as described before [30]. Enriched cysts were carefully collected from the interface, washed twice with ice-cold PBS, and kept at -20°C until DNA isolation.

2.5. Stool antigen detection

The Biomerica *Giardia* ELISA test (Biomerica Inc., Hannover, Germany) was performed on stool samples obtained from subjects and healthy controls according to the manufacturer's instructions. This kit is a stool antigen detection-based assay and is intended to detect specifically *Giardia* antigens in the stool. The well strips were measured in an automatic microtiter plate reader (BioTek ELX50, Winooski, Vermont, USA) at 450 nm. Stool samples with an optical density of 0.08 or greater after subtraction of the negative control optical density were considered as positive.

2.6. DNA preparation

Cyst walls of *G. duodenalis* were first disrupted by vortexing cyst suspensions in the presence of glass beads. Genomic DNA was subsequently isolated from trophozoites or

enriched cysts by using an AccuPrep® Stool DNA Extraction Kit (Bioneer, Daejeon, Korea). The concentration and purity of extracted DNA samples were measured by using a NanoDrop spectrophotometer (Thermo Fisher Scientific) and optical density (OD) ratio at 260/280 nm, respectively.

2.7. Semi-nested PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of the glutamate dehydrogenase (*gdh*) and triosephosphate isomerase (*tpi*) loci

PCR reactions were performed in 50 µl volume, containing 20 pmol of each forward and reverse *primers* and 25 µl of 2× Master Mix Red (Ampliqon, Copenhagen, Denmark), containing 1.5 mM MgCl₂. Primers used were External Forward Primer (Ampliqon, Copenhagen, Denmark) GDHeF (5'-TCA ACG TYA AYC GYG GYT TCC GT-3'), Internal Forward Primer GDHiF (5'-CAG TAC AAC TCY GCT CTC GG-3') and Reverse Primer GDHiR (5'-GTT RTC CTT GCA CAT CTC C-3') to specifically amplify a 432-bp fragment inside of the glutamate dehydrogenase (*gdh*) locus of *G. duodenalis* as described earlier [31]. To specifically amplify a 476-bp fragment inside of the triosephosphate isomerase (*tpi*) locus of the assemblage A (AI/AII) of *G. duodenalis*, primers used were External Forward Primer (Macrogen, Seoul, Korea) TPIAF (5'-CGAGACAAGTGTGAGATGC-3'), Internal Forward Primer TPIAIF (5'-CCAAGAAGGCTAAGCGTGC-3') and Reverse Primer TPIAR (5'-GGTCAAGAGCTTACAACACG-3') as described earlier (30). Also, primers used to specifically amplify a 140-bp fragment inside of the *tpi* locus of the assemblage B of *G. duodenalis* were External Forward Primer TPIBF (5'-GTTGCTCCCTCCTTTGTGC-3'), Internal Forward Primer TPIBIF (5'-GCACAGAACGTGTATCTGG-3') and Reverse Primer TPIBR (5'-CTCTGCTCATTGGTCTCGC-3') as described earlier [32].

For the PCR targeting the *gdh* locus, the amplification consisted of 30 cycles of 30 s at 94°C, 20 s at 60°C and 45 s at 72°C, with an additional final extension of 7 min at 72°C using a Flex Cycler PCR machine (Analytik Jena, Jena, Germany). In the second PCR, amplification consisted of 35 cycles, and the annealing temperature was increased to 62°C and other parameters were left unchanged. For the PCR targeting the *tpi* locus, the amplification consisted 25 cycles of 20 s at 94°C, 30 s at 52°C and 60 s at 72°C, with an additional final extension of 5 min at 72°C. In the second PCR, amplification consisted of 33 cycles, and the annealing temperature was increased to 56°C and other parameters were left unchanged.

Ten microliters of the PCR products resulting from the amplification of the *gdh* locus were digested with 5 U of *Bsp*LI (*Nla*IV; Fermentas, St. Leon-Rot, Germany) in 1× Tango buffer for 2 h at 37°C in order to distinguish between assemblages A (AI/AII) and B. Accordingly, the PCR products targeting the *tpi* locus were subjected to restriction enzyme digestion with *Rsa*I (Fermentas, St. Leon-Rot, Germany) in 1× Tango buffer for 2 h at 37°C in order to distinguish between genotypes AI and AII as described earlier [32]. The PCR products were separated on a 1-3% agarose gel (Takara Shuzo, Kyoto, Japan) in Tris-borate-EDTA buffer, stained with ethidium bromide (0.1 mg/ml), and visualized under UV light (UVItect, Cambridge, UK).

2.8. Sequence analysis of the *gdh* locus of *G. duodenalis* clinical isolates

For DNA sequencing, the secondary PCR products resulting from the amplifications of the *gdh* locus were gel-purified using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), and were sequenced directly using an ABI-3730XL DNA Analyzer capillary machine (Applied Biosystems, USA). DNA sequences were edited and aligned by Bio Edit version 7.2.5 and Sequence Scanner version 1.0 software, and compared with DNA sequences deposited in GenBank.

2.9. Phylogenetic analysis by maximum likelihoods of the *gdh* locus of *G. duodenalis* clinical isolates

Phylogenetic trees were constructed using Molecular Evolution Genetics Analysis software package version 6 (MEGA6) using DNA sequences acquired from the analysis of *G. duodenalis* parasites examined in the current study, compared with sequences from clinical isolates deposited in GenBank. DNA sequences of the *gdh* locus from the avian *G. ardeae* (AF069060) were used as the outgroup. The sequencing data were deposited in the GenBank and assigned the accession numbers KU565024-KU565030.

2.10. Cytokine analysis by ELISA

Peripheral blood specimens were collected from infected human subjects and healthy controls, serum was separated by centrifugation, and kept at -20°C until further analysis. Serum levels of cytokines were measured in duplicate by a sandwich ELISA method according to the manufacturer's instructions. Human ELISA kits for IFN- γ , IL-5 and IL-10 (eBioscience, Vienna, Austria) were used. The results were expressed as picograms of cytokine/mL serum (pg/mL). The sensitivity of ELISA kit was 0.99 pg/mL for IFN- γ , 1.5 pg/mL for IL-5 and 1 pg/mL for IL-10.

2.11. Statistical analyses

Data were analyzed using GraphPad Prism 6.07 software (GraphPad, San Diego, CA). For statistical analyses, a one-way ANOVA followed by Bonferroni post-hoc adjustment test for multiple comparison was employed. A two-tailed Fisher's exact test was used to calculate the association between symptomatic disease and the risk of infection with a given genotype. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Study population

We carried out a cross-sectional observational study, recruiting 38 healthy controls aged 2-30 and 40 human subjects with human giardiasis aged 3-34. Infected human subjects included 12 and 28 asymptomatic and symptomatic human subjects, respectively. Figure 1 delineates the clinical trial selection process. The demographic characteristics of healthy controls and human subjects infected with *G. duodenalis* are presented in Table 1. Asymptomatic human subjects had no apparent clinical manifestations, whereas symptomatic human subjects had symptoms of gastrointestinal disturbance (e.g. abdominal cramping) at the time of the diagnosis. Abdominal cramp was the most frequently complain

reported in human subjects with symptomatic giardiasis (Fig. 2A). Furthermore, symptomatic *G. duodenalis* infection was frequently associated with the soft stool consistency (67.9%), followed by formed (10.7%), loose (10.7%) and watery (10.7%) stool consistency (Fig. 2B).

3.2. The characterization of *G. duodenalis* clinical isolates based on *gdh* and *tpi* loci: predominance of assemblage A (AI) in both asymptomatic and symptomatic human subjects

To determine the genetic heterogeneity of *G. duodenalis* parasites isolated from asymptomatic and symptomatic human subjects, a 432-bp fragment within the *gdh* locus was amplified using primers GDHeF, GDHiF and GDHiR (Table S1). The PCR amplicons were the same size for both assemblages A and B parasites (Fig. 3A). After digestion with the restriction enzyme *Bsp*LI (*Nla*IV), the expected lengths of the amplified fragments were 90-, 120-, and 150-bp for the AI genotype and 70-, 80-, 90-, 120-bp for the AII genotype as shown in figure 3B [31].

Using primers TPIAF, TPIAIF, and TPIAR, a 476-bp fragment was amplified within the *tpi* of *G. duodenalis* clinical isolates (Fig. 3C). The subsequent digestion of the PCR products with *Rsa*I yielded in fragments of 437- and 15-bp for the AI genotype and 235-, 232-, and 15-bp for the AII genotype (Fig. 3D).

All isolates were successfully amplified at the *gdh* or *tpi* loci. As shown in figure 1, our analyses using both loci consistently showed that only 3 (7.5%) human subjects harbored assemblage B, whereas 37 (92.5%) human subjects were infected with assemblage A. Within assemblage A, 48.65% (18/37) were singly infected with AI genotype, whereas 27.03% (10/37) of infected human subjects had single AII infections. Moreover, 6 (16.22%) human subjects were found to be dually infected with AI and AII genotypes (mixed infections), whereas no mixed infections at intra-assemblage levels (A+B) were detected. However, 3 (8.1%) human subjects showed inconsistent electrophoretic patterns between the *gdh* and *tpi* loci and were excluded from further analysis. Single AI infections were found in 5 asymptomatic and 13 symptomatic human subjects, whereas single AII infections were detected in 3 and 7 asymptomatic and symptomatic human subjects, respectively. Using a two-tailed Fisher's exact test, no statistically significant differences were found between a given genotype and the presence of clinical symptoms.

3.3. *G. duodenalis* clinical isolates fell into three distinct clusters based of the *gdh* locus sequences

Our analysis identified a very low allelic sequence divergence within the isolates examined. Using the *gdh* locus from the avian *G. ardeae* as the outgroup to root the phylogenetic classification, phylogenetic analysis of aligned *gdh* locus sequences of *G. duodenalis* isolates obtained from human subjects singly infected with the genotypes AI or AII in the current study demonstrated the formation of distinct clusters (Fig. S1). Consistent with sequence data analyses, three different clusters were observed from which the first and the second clusters contained AI and AII genotypes, respectively. The other cluster represented

3 isolates belonging to assemblage B. Our analyses further showed the reliability of the *gdh* and *tpi* loci to separate common assemblages found in humans as shown previously [31, 33].

3.4. Cytokine profiles in asymptomatic and symptomatic human subjects

Distinct cytokine profiles at both the mRNA and protein levels have been demonstrated in asymptomatic and symptomatic parasitic diseases [34, 35]. We hypothesized that cytokine profiles in the sera of asymptomatic human subjects infected with *G. duodenalis* would be distinctive from that of symptomatic human subjects. We also hypothesized that varied immune profiles in human subjects infected with *G. duodenalis* are likely to be, in part, responsible for the variation observed in clinical outcomes of giardiasis in humans. In the current study, serum levels of IFN- γ , IL-5 and IL-10 were assessed using an ELISA method. Our analyses showed that serum levels of IFN- γ were significantly higher ($p < 0.05$) in the symptomatic group (14.02 ± 4.06 pg/mL) as compared to the asymptomatic group (6.3 ± 1.07 pg/mL) and healthy controls (4.81 ± 0.25 pg/mL). However, no significant differences were observed in the levels of serum IFN- γ between the asymptomatic group and healthy controls. Likewise, levels of both IL-5 and IL-10 were comparable between all the three groups (Fig. 4 and Table 2). Furthermore, no significant correlations were found between the parasite burdens, as determined by stool antigen detection assay and the absence or the presence of clinical manifestations (data not shown).

3.5. Human infection with the AI genotype of *G. duodenalis* is characterized by significantly higher levels of serum IFN- γ and IL-10 but not IL-5

We previously showed genotype-specific differences in the infection kinetics, immune responses, immunopathology as well as parasite-induced cytoskeletal changes in a mouse model of *G. duodenalis* [3, 8]. Considering the contribution of host factors to the development of clinical profiles of human giardiasis, we next hypothesized that parasite genotype would also contribute to the clinical outcomes of human giardiasis. Using ELISA, we tested whether genetically-diverse *Giardia* parasites could induce different serum cytokine profiles. Human subjects identified as being infected with assemblage B (3 subjects) were excluded from further analysis because the numbers were too small. As such, 6 human subjects detected as being dually infected with AI and AII genotypes as well as 3 human subjects with inconsistent electrophoretic patterns at both the *gdh* and *tpi* loci were also excluded from cytokine analysis. As shown in Figure 4, there were significantly higher levels of IFN- γ ($p < 0.05$) in the sera of human subjects infected with the AI genotype of *G. duodenalis* (14.65 ± 5.52 pg/mL) compared to human subjects infected with the AII genotype (6.4 ± 1.53 pg/mL) and healthy controls (4.81 ± 0.25 pg/mL). Likewise, human subjects infected with the AI genotype had significantly increased levels ($p < 0.01$) of serum IL-10 (3.33 ± 0.37 pg/mL) compared with human subjects with the AII genotype (1.64 ± 0.47 pg/mL) and healthy controls (1.97 ± 0.18 pg/mL). No statistically significant differences, however, were found in the serum levels of IL-5 in human subjects infected with the genotypes AI (11.52 ± 5.94 pg/mL), AII (6.34 ± 1.58 pg/mL) or healthy controls (5.57 ± 0.33 pg/mL). These findings suggested that genetically-dissimilar isolates of *G. duodenalis* might behave differently in human host by inducing distinct signatures of immune responses.

4. Discussion

In the current study, we have tested host (i.e. serum levels of IFN- γ , IL-5, IL-10) and parasite factors (parasite genetic heterogeneity) contributing to the development of clinical symptoms of human giardiasis. We found that the AI genotype of *G. duodenalis* was the dominant parasite identified in both asymptomatic and symptomatic human subjects in an endemic area of human giardiasis, followed by the AII genotype. Increased levels of the proinflammatory cytokine IFN- γ , but not IL-5 or IL-10, were detected in the sera of symptomatic human subjects infected with *G. duodenalis*. No statistical differences in serum levels of cytokines were observed in asymptomatic cyst passers compared with healthy controls. Furthermore, human subjects infected with the AI genotype of *G. duodenalis* showed elevated serum levels of the cytokines IFN- γ and IL-10 but not IL-5, whereas human subjects infected with the AII genotype and healthy controls had comparable levels of those cytokines in the serum.

Several mechanisms have been suggested as to how the lumen-dwelling parasite *G. duodenalis* can elicit immune responses (reviewed in [3, 36]). The intestinal barrier dysfunction and increased intestinal permeability have been shown to occur following infection with *G. duodenalis* [37]. The lack of an intact intestinal barrier may facilitate the translocation of exogenous parasite antigens into the underneath layers of the intestine, inducing the lamina propria [37]. This, in turn, may account for the induction of pro-inflammatory response within the intestine. Alternatively, the direct invasion of the intestinal mucosa by some strains of *G. duodenalis in vivo* may explain how immune response are elicited by a lumen-dwelling intestinal parasite [38].

Elevated levels of IFN- γ have been reported during human and murine giardiasis, suggesting a protective role for this cytokine in host defense against *G. duodenalis* infection [8, 14, 15, 39, 40]. However, mice deficient in IFN- γ (IFN- $\gamma^{-/-}$) were able to eventually control the *Giardia* infection later in the course of infection in a manner similar to their WT littermates [17], suggesting a potential protective role for this cytokine early in the course of infection. Despite the potential protective roles that may be played by IFN- γ during microbial infections, higher levels of IFN- γ have been linked to immunopathology following infection in a wide range of microbial models [41, 42]. For example, infection with *Toxoplasma gondii* induced a fatal CD4⁺ T cell- and IFN- γ -dependent inflammatory response in the intestine of infected C57BL/6 mice [43]. Likewise, a strong correlation was found between facilitated egg-induced immunopathology and elevated levels of IFN- γ in a murine model of the blood fluke *Schistosoma mansoni* [41]. We found that IFN- γ , IL-5 and IL-10 were produced following human giardiasis, irrespective of clinical profile, and that only human subjects with symptomatic *G. duodenalis* infections showed statistically higher levels of IFN- γ , but not IL-5 and IL-10 compared to healthy controls. Comparable levels of the later cytokines, however, were found in healthy controls and human subjects with asymptomatic giardiasis. These findings may highlight roles played by host factors in defining the clinical outcomes during human infection with *G. duodenalis* and may also further explain, in part, why only a small fraction of human subjects infected with this parasite manifest symptomatic disease, whereas the majority do not show any overt symptoms of the infection [3].

Next we tested whether parasite genotype contributed to varied clinical outcomes of human giardiasis. It is known that genetically-distinct parasite strains differ in their virulence capacities in numerous host-parasite systems, the ability to induce pathological changes as well as the way they elicit immune responses within mammalian hosts [44-49]. We found that human subjects infected with the AI genotype of *G. duodenalis* had significantly higher serum levels of IFN- γ and IL-10, but not IL-5, compared with healthy controls. Conversely, no statistically significant differences were observed in the serum levels of those cytokines in human subjects infected with the AII genotype of the parasite compared to healthy controls. Genetically-diverse *G. duodenalis* assemblages have been demonstrated to possess varied capabilities to colonize the small intestine *in vivo* [8]. Although these differences have yet to be shown to occur at inter-assemblage levels (AI vs. AII), they may, in part, explain why human subjects infected with genotypes AI and AII show differential expression of serum cytokines. Collectively, these results are consistent with earlier observations suggesting that genetically-diverse *Giardia* parasites may differ considerably in their propensity to induce immunopathology and tissue damages [8, 45].

Taken together, our findings underscore the notion that both host and parasite factors may play key roles in determining the outcomes of human giardiasis, and may further explain why only a small portion of infected human subjects develop symptomatic disease, whereas the majority do not. However, the causality, the direction of causality and the potential mechanisms underlying the differences in the cytokine profiles between asymptomatic and symptomatic human subjects may warrant further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig 1.

Flow diagram deciphering the subject selection process. This diagram outlines the study recruitment of 40 subjects who were stratified into different groups based on the absence or the presence of clinical manifestations and the presence of infection with a particular genotype of *G. duodenalis* (AI/AII). Genotypes analyzed do not add up to 40 as some genotypes ($n= 3$) were excluded from cytokine analysis because the results obtained between the *gdh* and *tpi* loci were inconsistent.

Fig 2. Breakdown of clinical symptoms in human subjects ($n = 28$) with symptomatic giardiasis (A). Fecal consistency in human subjects with human giardiasis was determined by a scoring system, where scores of 1 to 4 denoted formed, soft, loose, and watery stool consistency, respectively (B).

Fig 3.

Agarose gel electrophoresis of the semi-nested PCR amplification of a 432-bp of the *gdh* gene of *G. duodenalis*. Lanes 1, negative control (no DNA); 2, positive control; 3-5, patient's samples; M, DNA ladder (A). Digestion of PCR product targeting the *gdh* gene using *Bsp*LI (*Nla*IV) to distinguish AI (90-, 120-, 150-bp) and AII (70-, 80-, 90-, 120-bp) genotypes. Lane 1, undigested PCR product; 2 and 3, AI genotype; 4 and 5, AII genotype; 6, mixed infections (AI+AII); M, DNA ladder (B). Agarose gel electrophoresis of the semi-nested PCR amplification of a 476-bp of the *tpi* gene of the *G. duodenalis* assemblage A. Lanes 1, positive control; 2-6, patient's samples; 7, negative control (no DNA); M, DNA ladder (C). Digestion of PCR product targeting the *tpi* gene using *Rsa*I to distinguish AI (437-, 15-bp) and AII (232-, 235-, 15-bp) genotypes. Lane 1, undigested PCR product of the *G. duodenalis* assemblage A; 2-4, AI genotype; 5, AII genotype; 6 and 7, mixed infections (AI+AII); M, DNA ladder (D). Sizes in base pairs are denoted by the numbers at left.

Fig 4.

Cytokine profiles in asymptomatic and symptomatic human giardiasis and in human subjects infected with genetically-distinct *G. duodenalis* genotypes. Human subjects infected with *G. duodenalis* were stratified into asymptomatic and symptomatic patients based on the absence or the presence of clinical symptoms. Serum levels of the cytokines IFN- γ (A), IL-5 (B) and IL-10 (C) were measured using a sandwich ELISA method in human subjects with asymptomatic ($n = 12$) and symptomatic ($n = 28$) giardiasis as well as in healthy controls ($n = 38$). * $p < 0.05$ (a one-way ANOVA, followed by Bonferroni post-hoc adjustment test). Bar

graphs represent Mean $\pm$ SEM. Clinical isolates of *G. duodenalis* from asymptomatic and symptomatic patients were genetically characterized by a semi-nested PCR targeting the *gdh* or *tpi* loci, followed by a restriction fragment length polymorphism (RFLP) method. Serum levels of IFN- γ (D), IL-5 (E), and IL-10 (F) were measured in human subjects singly infected with AI ($n=18$) or AII ($n=10$) genotypes as well as in healthy controls ($n=38$) by ELISA. * $p < 0.05$; ** $p < 0.01$ (A one-way ANOVA, followed by Bonferroni post-hoc adjustment test). Bar graphs represent Mean $\pm$ SEM.

Table 1

Study group demographics

	Age (y)			Sex	
	Median	Mean $\pm$ SEM	Range	Female (%)	Male (%)
Healthy Controls (<i>n</i> =38)	8	11.61 $\pm$ 1.31	2-30	20 (52.6)	18 (47.4)
Asymptomatic (<i>n</i> =12)	19	18.75 $\pm$ 2.51	5-29	4 (33.3)	8 (66.7)
Symptomatic (<i>n</i> =28)	8.5	11.46 $\pm$ 1.68	3-34	12 (42.8)	16 (57.2)

Table 2

Serum concentrations of cytokines in individuals participated in this study

Cytokine (pg/mL)	Median	Mean±SEM	Range	<i>p</i> Value
Healthy Controls (n=38)				
IFN- γ	4.61	4.81±0.25	1.36-9.16	-
IL-5	5.02	5.57±0.33	0.00-9.80	-
IL-10	2.13	1.97±0.18	0.00-3.98	-
Asymptomatic (n=12)				
IFN- γ	4.79	6.30±1.07	3.48-14.79	-
IL-5	3.89	7.43±1.86	1.62-22.01	-
IL-10	2.50	2.64±0.30	1.15-4.88	-
Symptomatic (n=28)				
IFN- γ	5.65	14.02±4.06	1.36-88.53	$p<0.05^a$
IL-5	4.08	9.71±3.86	0.00-110.3	<i>n.s.</i>
IL-10	2.51	2.58±0.33	0.00-8.04	<i>n.s.</i>
Genotype AI (n=18)				
IFN- γ	5.16	14.65±5.52	3.48-88.53	$p<0.05^a$
IL-5	3.58	11.52±5.94	0.00-110	<i>n.s.</i>
IL-10	3.07	3.33±0.37	1.54-8.04	$p<0.001^b$
Genotype AII (n=10)				
IFN- γ	4.90	6.40±1.53	1.36-17.17	-
IL-5	4.20	6.34±1.58	1.63-16.54	-
IL-10	1.35	1.64±0.47	0.00-3.98	-

^a as compared to healthy controls^b as compared to healthy controls and AI vs. AII