

Association between TNF- α and *Entamoeba histolytica* Diarrhea

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Abstract. An association between tumor necrosis factor α (TNF- α) and *Entamoeba histolytica* diarrhea was assessed in a cohort of 138 non-related Bangladeshi children who have been prospectively followed since 2001. Peripheral blood mononuclear cells (PBMCs) obtained at study entry were purified, cultured, and stimulated with soluble amebic antigen before cytokine measurement from supernatant. Higher levels of TNF- α were associated with increased risk of first ($P = 0.01$) and recurrent *E. histolytica*-related diarrheal episodes ($P = 0.005$). Children who developed *E. histolytica* diarrhea had significantly higher TNF- α protein levels than those who experienced asymptomatic *E. histolytica* infection (P value = 0.027) or no infection (P value = 0.017). Microarray studies performed using RNA isolated from acute and convalescent whole blood and colon biopsy samples revealed higher but non-significant TNF- α messenger RNA (mRNA) levels in subjects with acute *E. histolytica* diarrhea compared with convalescence. We conclude that there is an association between higher TNF- α production and *E. histolytica* diarrhea.

INTRODUCTION

Amebiasis, caused by *Entamoeba histolytica*, is a disease of worldwide importance and estimated to result in 40,000–100,000 deaths annually. Because the primary mode of *E. histolytica* transmission is through ingesting fecally contaminated food and water, its prevalence is greatest in areas with inadequate sanitation.¹ The majority of infections with *E. histolytica* are asymptomatic, but more than 10% of infections result in diarrhea.² Importantly, *E. histolytica* diarrhea in children is associated with malnourishment and stunting.³

Both increased levels of interferon-gamma (IFN- γ) from stimulated peripheral blood mononuclear cells (PBMCs)⁴ and a mucosal IgA antilectin antibody response⁵ have been previously associated with protection from *E. histolytica* infection. However, much information is still unknown about the aspects of the immune response that determine whether an individual with an *E. histolytica* infection remains asymptomatic or develops disease. Several *in vitro* and animal studies have evaluated the effect of tumor necrosis factor α (TNF- α) on *E. histolytica* with mixed results. It has been shown to both increase *E. histolytica* killing and worsen tissue damage.^{6–16} Therefore, the impact of TNF- α on disease outcome is still not clear.

The TNF- α is a pro-inflammatory cytokine, largely produced by macrophages, which can lead to tissue inflammation through the activation of macrophages, recruitment of neutrophils, and up-regulation of other pro-inflammatory mediators.¹⁷ It can also increase cell permeability, resulting in impairment of barrier function and edema formation.¹⁸ The TNF- α plays a central role in mucosal inflammation, and is elevated in the gastrointestinal tract of some forms of inflammatory colitis.¹⁹ For example, this cytokine is known to be of significant importance in the pathogenesis of inflammatory bowel disease (IBD), and anti-TNF agents have proven to be effective treatment of some individuals.²⁰ In addition, stool TNF- α concentrations are higher in acute shigellosis compared with other forms of viral or bacterial diarrhea.^{21,22}

This study tested the hypothesis that TNF- α promotes *E. histolytica* diarrhea. The PBMCs were tested for their ability to produce TNF- α in response to amebic antigen, and the association of TNF- α levels with susceptibility to invasive amebiasis measured. In addition, immunohistochemical (IHC) staining of colon biopsy samples and TNF- α messenger RNA (mRNA) levels in colon and whole blood were conducted.

MATERIALS AND METHODS

Study population. Cytokine levels were measured from the supernatant of stimulated PBMCs collected from 138 non-related children in the area of Mirpur within Dhaka, Bangladesh, between October 2001 and August 2002. After blood collection for cytokine analysis, the children were followed for an average of 1,785 days (SD \pm 513 days). Monthly stool specimens were routinely obtained from all children in the cohort and tested for multiple pathogens, including *E. histolytica*. The children were also interviewed within their homes every other day, and stools were collected when diarrhea was reported. Height and weight of the children were obtained by research assistants at study enrollment and then every 4 months. Measurements obtained closest to the blood draw were used in analysis of this study.

The RNA for microarray analysis was isolated from whole blood obtained from seven children of the Mirpur cohort with acute *E. histolytica* diarrhea, and again 30 days later during convalescence. Colon biopsy samples for microarray analysis and IHC were obtained from eight subjects, ranging in age from 17 to 40, who came to the International Center for Diarrheal Diseases, Bangladesh with acute *E. histolytica* diarrhea. Biopsy samples were also collected 60 days later during convalescence.

Diagnosis of *E. histolytica* infection. *Entamoeba histolytica* infection was determined using the *E. histolytica* II test (TechLab, Inc., Blacksburg VA), which detects amebic antigen in stool. A new *E. histolytica* infection was defined as a positive *E. histolytica* stool antigen and/or culture result following > 2 months of negative surveillance stool samples. *Entamoeba histolytica* diarrhea was defined as three or more unformed stools in a 24-hour period in a child diagnosed with a new episode of *E. histolytica* infection.

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Measurement of secreted cytokines. After isolation from whole blood, PBMCs were cultured in the presence of soluble amebic abstract (SAE) or phytohemagglutinin (PHA) for 72 hours. The resulting supernatant was frozen at -70°C until further use. Cytokine levels were subsequently measured from supernatant using a Bio-Plex human cytokine 17-plex assay (interleukin [IL]-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, TNF- α , IFN- γ , granulocyte colony-stimulating factor [G-CSF], granulocyte macrophage colony-stimulating factor [GM-CSF], monocyte chemoattractant protein-1 [MCP-1], and macrophage inflammatory protein-1 β [MIP-1 β]) (Bio-Rad, Hercules, CA), according to manufacturer's instructions.

Microarray. The RNA for microarray analysis was isolated from whole blood collected into Tempus tubes (Applied Biosystems, Warrington, UK) from seven children of the Mirpur cohort with acute *E. histolytica* diarrhea, and again 30 days later during convalescence. The Tempus spin RNA Isolation Reagent Kit (Applied Biosystems) was used to isolate blood from Tempus tubes per manufacturer's instructions.

Colon biopsy samples for microarray analysis were obtained from eight subjects, ranging in age from 17 to 40, who came to the International Center for Diarrheal Diseases, Bangladesh with acute *E. histolytica* diarrhea. Biopsy samples were also collected 60 days later during convalescence. Samples of colon tissue were homogenized in Trizol (Invitrogen, Carlsbad, CA), and RNA was isolated using the PicoPure RNA Isolation Kit (Molecular Devices, Sunnyvale, CA).

Microarray processing. Samples were prepared according to Affymetrix protocols (Affymetrix, Inc., Santa Clara, CA). The RNA quality and quantity was ensured using the Bioanalyzer (Agilent, Inc., Santa Clara, CA) and NanoDrop (Thermo Scientific, Inc., Wilmington, DE), respectively. Per RNA labeling, 200 nanograms of total RNA was used in conjunction with the Affymetrix recommended protocol for the GeneChip 1.0 ST chips. The hybridization cocktail containing the fragmented and labeled complementary DNAs (cDNAs) was hybridized to the Affymetrix Human Genome ST 1.0 GeneChip. The chips were washed and stained by the Affymetrix Fluidics Station using the standard format and protocols as described by Affymetrix. The probe arrays were stained with streptavidin phycoerythrin solution (Molecular Probes, Carlsbad, CA) and enhanced by using an antibody solution containing 0.5 mg/mL of biotinylated anti-streptavidin (Vector Laboratories, Burlingame, CA). An Affymetrix Gene Chip Scanner 3000 was used to scan the probe arrays. Gene expression intensities were calculated using the GeneChip Operating software version 1.2 (GCOS 1.2, Affymetrix). The robust microarray analysis (RMA) within the Affymetrix Expression Console was used to normalize and log-transform the data and generate the .CHP files.

Immunohistochemical staining. The IHC was performed for the detection of TNF- α in three colon biopsy samples obtained from subjects during an acute episode of *E. histolytica* diarrhea, and again at 60 days after recovery. Subjects with acute disease were stool antigen positive for *E. histolytica*, had blood and mucous in their stools, and were culture negative for other common diarrheal agents. Tissue sections were deparaffined and rehydrated before antigen retrieval using the Pascal Pressure Chamber with TRS buffer (DAKO, Denmark) for 30 seconds at 125°C and 22 psi. Slides were stained using the Dako autostainer Universal Staining system (DAKO). Sections were incubated for 10 minutes with DAKO Dual Endogenous

Enzyme Block before 30-minute incubation with the primary antibody, TNF- α (dilution 1:200). Sections were subsequently incubated for 30 minutes with DAKO Envision Dual Link (anti-mouse, rabbit antibody) before incubation with chromagen substrate diaminobenzidine tetrahydrochloride (DAB) for 10 minutes. The slides were counterstained with hematoxylin and mounted. Negative controls were performed by omitting the primary antibody. Colon tissue from subjects with ulcerative colitis was used as a positive control.

Statistical analysis. Time to first event (*E. histolytica* asymptomatic infection and/or diarrhea) analysis was modeled for all cytokines using the proportional hazard model and found to be significant for TNF- α from SAE stimulated supernatant and *E. histolytica* diarrhea. To concisely model the association between the risk of *E. histolytica* diarrhea and TNF- α levels, analysis of recurrent events was performed. The inference was based on the proportional means model with the robust sandwich covariance estimate,²³ which is an analogue to the Cox proportional hazards model. To examine the potential confounding effect of nutritional status, the nutrition score weight for height z-score (WHZ) closest to the blood draw was included in the model. In all of the previous survival analysis models, the continuous level of TNF- α protein was used. To illustrate the magnitude of TNF- α effect on the risk of *E. histolytica* diarrhea, each 1,000 pg/mL increment of TNF- α was used to calculate hazard ratio. One-way analysis of variance (ANOVA) was used to examine the difference in TNF- α protein expression from supernatant among children with a history of *E. histolytica* diarrhea, asymptomatic infection, or no infection. Two-sample *t* tests were used to compare differences between the two groups.

Microarray analysis. The Genesifter program (Geospiza, Inc., Seattle, WA) (<http://www.genesifter.net/web/>) was used for analysis. A pairwise *t* test was used to compare TNF- α mRNA levels from acute and convalescent whole blood and colon samples.

Informed consent was obtained from adult participants and from the parents or legal guardians of minors. The studies were reviewed and approved by the Institutional Review Board at the University of Virginia and the Ethical Review Committee of the International Center for Diarrheal Disease Research, Bangladesh, Dhaka, Bangladesh.

RESULTS

Association with survival. The minimum value of TNF- α was 0 pg/mL, median 1,550 pg/mL, and maximum 14,915 pg/mL. Of the 138 non-related children included in this analysis, there were 107 new *E. histolytica* infections during the observation period, of which 25 children had diarrhea/colitis. Higher levels of TNF- α from SAE stimulated supernatant were associated with increased risk of first diarrheal episode. With each 1,000 pg/mL increase of TNF- α , the chance to have a first episode of diarrhea was elevated by 18% (hazard ratio = 1.18, 95% confidence interval [CI]: 1.04, 1.33, $P = 0.01$). The hazard ratio for subjects with a median TNF- α level (1,550 pg/mL) compared with those with a minimum TNF- α level (0 pg/mL) was 1.28 (95% CI: 1.06, 1.56). Figure 1 shows the steady increase of 5-year diarrhea rate (percentage of children who had diarrhea) with TNF- α level, based on the previous survival analysis model. The average 5-year diarrhea rate for children with minimal TNF- α level (0 pg/mL) is 13%,

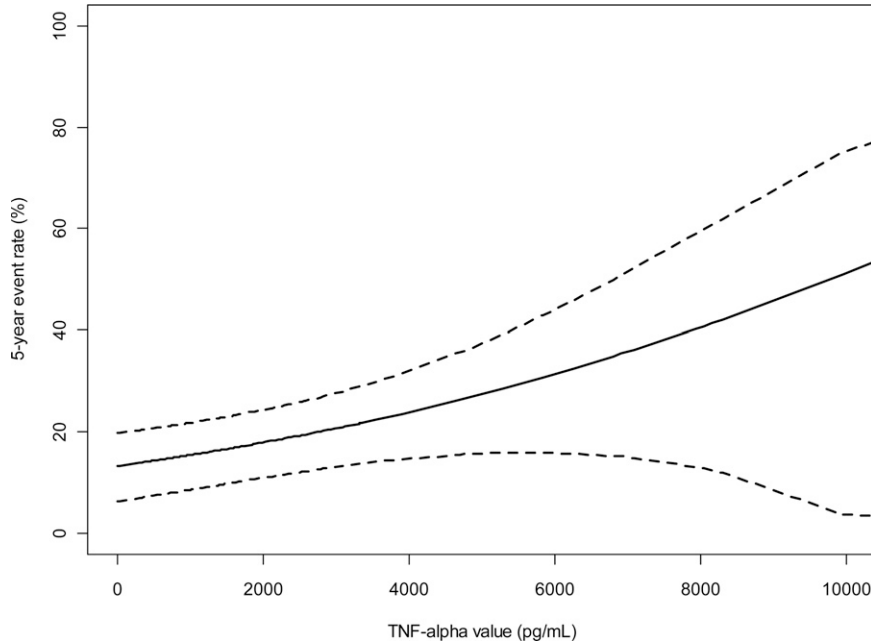


FIGURE 1. Five-year event rate for *Entamoeba histolytica* diarrhea based on TNF- α levels. Analysis of time to first event showed that with each 1,000 pg/mL increase of TNF- α , the chance to have *E. histolytica* diarrhea was elevated by 18% (hazard ratio = 1.18 and 95% confidence interval: 1.04, 1.33, $P = 0.001$).

whereas it is 17% for those with median TNF- α (1,550 pg/mL). When TNF- α rises to 5,000 pg/mL, the 5-year diarrhea rate elevates to 27%.

After adjusting for co-infection with the soil-transmitted helminthes *Ascaris lumbricoides* or *Trichuris trichuria*, the association between TNF- α and time to first *E. histolytica* diarrheal episode remained significant ($P = 0.01$ and $P = 0.02$, respectively) and the hazard ratio estimates remained almost unchanged. In contrast, time to first *E. histolytica* asymptomatic or total infections were not associated with TNF- α levels ($P = 0.30$ and $P = 0.15$, respectively, NS). No other measured cytokine was associated with time to *E. histolytica* infection or diarrhea, and there was no association with TNF- α from PHA stimulated supernatant and time to *E. histolytica* diarrheal episode ($P = 0.35$). Therefore, to determine if the TNF- α responses reflected prior amebic-specific T-cell acquired memory, we additionally adjusted for baseline serum anti-*E. histolytica* antibodies. The association between TNF- α and time to first *E. histolytica* diarrheal episode also remained significant ($P = 0.01$).

There were 45 total episodes of diarrhea, with 12 subjects having recurrent episodes of *E. histolytica* diarrhea (Table 1). Higher levels of TNF- α from SAE stimulated supernatant were associated with increased risk of recurrent *E. histolytica*-related diarrheal episodes ($P = 0.005$). Analysis of recurrent events showed that with each 1,000-pg/mL increase of TNF- α , the chance to have *E. histolytica* diarrhea was elevated by 14% (hazard ratio = 1.14 and 95% CI: 1.04, 1.26, $P = 0.005$). The hazard ratio for subjects with a median TNF- α level (1,550 pg/mL) compared with those with a minimum TNF- α level (0 pg/mL) was 1.23 (95% CI: 1.07, 1.43). The TNF- α levels from PHA stimulated supernatant were not associated with recurrent episodes of *E. histolytica* diarrhea ($P = 0.42$).

There were 617 total episodes of diarrhea from any cause among the 138 children in the cohort since 2001. To determine

if the association with TNF- α was specific for *E. histolytica* or a general response caused by diarrhea itself, a recurrent event analysis was performed using all causes of diarrhea in the cohort since 2001. The TNF- α was not associated with total all cause diarrheal episodes (hazard ratio = 1.03, $P = 0.38$).

Nutritional status. For recurrent event analysis, after adjusting for WHZ, the association between *E. histolytica* diarrhea and TNF- α levels remained significant ($P = 0.003$). Therefore, the association of TNF- α with *E. histolytica* diarrhea was independent of nutritional status.

Comparison of protein expression. The TNF- α protein expression from supernatants of the SAE stimulated PBMCs obtained at study entry was compared among three groups of children: those who have never been infected with *E. histolytica*; children who have had asymptomatic *E. histolytica* infection; and children who have had *E. histolytica* diarrhea. Differences were seen when comparing all three groups using ANOVA ($P = 0.04$) (Table II). Children with *E. histolytica* diarrhea had significantly higher TNF- α protein levels than those with asymptomatic *E. histolytica* infection (P value = 0.027) and those with no *E. histolytica* infection (P value = 0.017) (Table II). Children with *E. histolytica* diarrhea also had significantly higher TNF- α protein levels than the combined

TABLE 1

Number and frequency of episodes of *Entamoeba histolytica* diarrhea among the 138 subjects in whom TNF- α levels from supernatant were obtained

Number of episodes of <i>E. histolytica</i> diarrhea	Number of children	Percent of all children with an episode of <i>E. histolytica</i> diarrhea
1	13	52
2	9	36
4	2	8
6	1	4

TABLE 2

Comparison of TNF- α protein expression from supernatant of SAE stimulated PBMCs among children with a history of asymptomatic infection or no history of infection compared with symptomatic *Entamoeba histolytica* infection with diarrhea*

	N	Mean (SEM)	P value
TNF- α protein (pg/mL)			
No <i>E. histolytica</i> infection	31	1697 (377)	0.017
Asymptomatic	82	1988 (232)	0.027
<i>E. histolytica</i> infection			
Symptomatic	25	3061 (420)	–
<i>E. histolytica</i> infection			

*The global P value = 0.04. SAE = soluble amebic abstract; PBMCs = peripheral blood mononuclear cells; SEM = standard error of the mean.

groups of children with asymptomatic *E. histolytica* infection and no *E. histolytica* infection (P value = 0.011). There was no difference between children asymptotically infected and never infected (P value = 0.51).

Comparison of acute and convalescent mRNA expression.

The mRNA expression was higher in acute (7.22, standard error of the mean [SEM] 0.10) compared with convalescent (7.02, SEM 0.06) whole blood samples, and acute (6.09, SEM 0.10) compared with convalescent (5.98, SEM 0.06) colon biopsy samples, but the differences were non-significant (P = 0.13 and 0.34, respectively).

Immunohistochemistry. The TNF- α stained mononuclear cells in the lamina propria with abundant cytoplasm consistent with macrophages. No differences were observed in TNF- α staining between acute and convalescent amebic colitis (Figure 2).

CONCLUSIONS

Although the majority of *E. histolytica* infections are asymptomatic, amebiasis remains a significant cause of morbidity and mortality in areas of the world where its prevalence is high. The host factors that influence disease development in *E. histolytica* infections have not been fully elucidated. This study evaluated the role of cytokines, and found an association between protein TNF- α levels from supernatant of SAE stimulated PBMCs and susceptibility to *E. histolytica* disease. Higher levels of TNF- α were associated with increased risk of first and recurrent *E. histolytica*-related diarrheal episodes, suggesting production of TNF- α may predict future susceptibility to *E. histolytica* diarrhea. This association was not seen with diarrhea in general from all causes. We also found no association with baseline serum anti-*E. histolytica* antibodies, suggesting an association with differential innate immune responses rather than prior amebic-specific T-cell acquired memory. Consistent with this, Chadee and others¹³ have demonstrated the *E. histolytica* adherence lectin (Gal-lectin) stimulates TNF- α by macrophages.

Elevated PBMC TNF- α protein expression was seen in children who had *E. histolytica* diarrhea compared with those who had asymptomatic infection or no infection. Higher although non-significant TNF- α mRNA expression among whole blood and colonic biopsy samples from subjects with acute versus convalescent *E. histolytica* diarrhea was also noted. These findings further suggest a role of TNF- α in the susceptibility to and pathogenesis of *E. histolytica* diarrhea. In agreement with these results, a study using human colonic xenografts reported that genes activated by TNF- α (IL-1 β , IL-6, or IL-8) had increased expression during *E. histolytica* infection compared with control colonic samples without infection.¹⁶

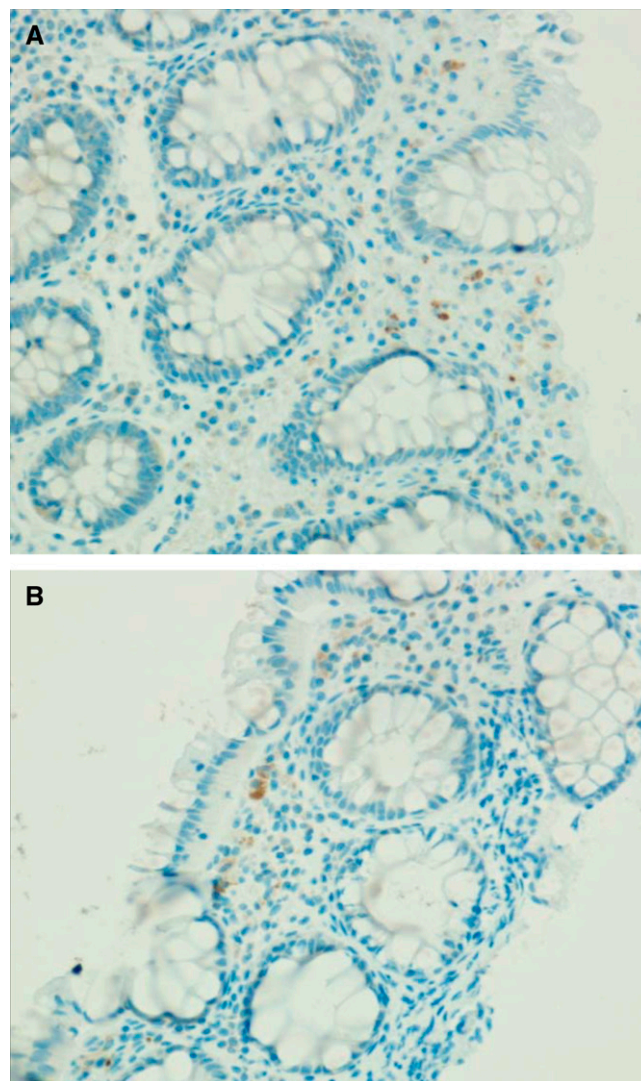


FIGURE 2. TNF- α stained mononuclear cells in the lamina propria with abundant cytoplasm consistent with macrophages in **A**, human colon biopsy on Day 1 of an *Entamoeba histolytica* diarrhea episode and **B**, Day 60 after recovery from *E. histolytica* diarrhea. This figure appears in color at www.ajtmh.org.

Obvious differences in TNF- α staining by IHC were not apparent between acute *E. histolytica* diarrhea and convalescent colonic biopsy samples; however, the sample size, three subjects, was small. The biopsy samples also represent a very limited area of the colon, and may have not assessed areas with greater TNF- α variability. Furthermore, methods that quantify differences in TNF- α levels may be more sensitive.

Previous studies have shown conflicting results regarding the role of TNF- α and amebae. Some *in vitro* studies have shown that TNF- α leads to increased nitric oxide (NO) production and killing of ameba.^{8,10-12} These findings would suggest that TNF- α may be beneficial in amebic colitis. In contrast, TNF- α has been shown to worsen tissue damage in mouse models of amebic colitis.^{15,16} The TNF- α also attracts *E. histolytica* trophozoites through chemotaxis,⁷ which may aid the parasite in the process of tissue invasion and colitis. On the basis of the current studies' findings, TNF- α does not appear to be protective for development of *E. histolytica* diarrhea and may actually increase susceptibility.

The TNF- α has been shown to play an important role in the pathogenesis of other gastrointestinal disorders such as IBD. Several studies have shown increased TNF- α levels in the serum and intestinal mucosa of individuals with IBD,^{24,25} and TNF- α blocking agents have been shown to result in healing of tissue inflammation in some patients. In addition to the direct effects of TNF- α itself on tissue, it may also lead to the upregulation of other pro-inflammatory cytokines and matrix metalloproteinases (MMPs). The MMPs are a family of Ca- and Zn-activated endoproteinases that are involved in physiological turnover, and mediate degradation of the extracellular matrix.²⁶ In IBD patients, MMP-1, 3, 7, 9, and 10 are significantly increased in inflamed compared with normal colonic mucosa.²⁷ Both MMPs and tissue inhibitor matrix metalloproteinases (TIMPs) are affected by TNF- α ²⁶; TNF- α increases the expression of MMP-3, MMP-9, and MMP-10.^{27,28} We have similarly seen significantly increased levels of MMPs in both colonic and PBMC mRNA expression among those with acute *E. histolytica* diarrhea compared with convalescence (Peterson K, Duggal P, Haque R, et al., unpublished data).

Environmental, pathogen, and host factors may each contribute to the outcome of *E. histolytica* infections. We have examined environmental factors including nutritional status and the contribution of co-infection with soil-transmitted helminthes. The effect of TNF- α was found to be independent. Pathogen factors, such as different parasite genotypes, could also influence the susceptibility to disease, potentially through different degrees of TNF- α stimulation. Finally, host genetic polymorphisms, such as in TNF- α or its pathways, may lead to variation in individual immune responses and contribute to the differences seen in disease susceptibility as well.

In conclusion, this study found an association between higher TNF- α production and *E. histolytica* diarrhea. An over-aggressive immune response from TNF- α may lead to increased inflammation and therefore disease. Further studies are warranted to clarify the role of TNF- α and its pathways in amebic colitis.

Received August 25, 2009. Accepted for publication December 24, 2009.

Acknowledgments: We thank the families in Mirpur, Dhaka Bangladesh for their participation in these studies. We also thank Pat Pramoonjago and Christopher A. Moskaluk for assistance with immunohistochemistry.

Financial support: This work is supported by NIH grant K08A1072470 to Kristine M. Peterson, grant AI043596 to William A. Petri, and in part by the intramural division of the National Human Genome Research Institute, NIH.

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