CORRELATION OF INTERFERON-\(\gamma\) PRODUCTION BY PERIPHERAL BLOOD MONONUCLEAR CELLS WITH CHILDHOOD MALNUTRITION AND SUSCEPTIBILITY TO AMEBIASIS

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Abstract. The contribution of interferon-\(\gamma\) (IFN-\(\gamma\)) to immunity from amebiasis was assessed in a three-year prospective study of children 2–5 years of age in an urban slum of Dhaka, Bangladesh. IFN-\(\gamma\) produced by peripheral blood mononuclear cells stimulated with soluble amebic antigen was measured upon enrollment. Thirty-one of the 209 enrolled children had Entamoeba histolytica–associated diarrhea. Children who produced higher than the median level of IFN-\(\gamma\) (median = 580 pg/mL) had longer survival without E. histolytica diarrhea/dysentery (log rank test \(P = 0.03\)) and a reduction in the risk of E. histolytica diarrhea/dysentery by more than half (Cox proportional hazard regression = 0.45, \(P = 0.04\)). When adjusted for stunting, the association between IFN-\(\gamma\) and the time to the first episode of E. histolytica-associated diarrhea remained marginally significant (Cox proportional hazard regression = 0.49, \(P = 0.07\)). We conclude that production of IFN-\(\gamma\) is linked to nutritional status and predicts future susceptibility to symptomatic amebiasis.

INTRODUCTION

Amebic colitis and liver abscesses are caused by infection with the extracellular protozoan parasite Entamoeba histolytica.1–4 Carefully conducted serologic studies in Mexico, where amebiasis is endemic, demonstrated antibody to E. histolytica in 8.4% of the population.5 In the urban slum of Fortaleza, Brazil, 25% of the people tested had antibody to E. histolytica; the prevalence of anti-amebic antibodies in children 6–14 years of age was 40%.6 Our prospective study of preschool children in a slum of Dhaka, Bangladesh showed new E. histolytica infections in 45%, and E. histolytica-associated diarrhea in 9% of children annually.7 Long-term consequences of amebiasis may include both malnutrition8 and lower cognitive abilities.9

An understanding of the nature of protective immunity could provide a rational framework for the design of an amebiasis vaccine. Earlier work had demonstrated that acquired immunity to amebiasis is linked to an IgA antibody response against the carbohydrate recognition domain of the parasite Gal/GalNAc lectin.7.10–12 The protection associated with IgA to carbohydrate recognition domain was partial, lasting slightly more than one year.

In contrast, the role of cell-mediated immunity in protection from amebiasis has not been delineated and is the subject of this study. There is substantial evidence from in vitro and animal model studies of an important role for interferon-\(\gamma\) (IFN-\(\gamma\)) including IFN-\(\gamma\) activation of macrophages to kill the parasite,13–17 increased susceptibility to amebiasis in IFN-\(\gamma\)–deficient mice or upon neutralization of IFN-\(\gamma\),16–20 and correlation of vaccine-induced protection with antigen-specific production of IFN-\(\gamma\) in the gerbil model of amebic liver abscess.21,22 Two studies with small numbers of patients have suggested an association between IFN-\(\gamma\) and protection from E. histolytica disease. Two individuals with asymptomatic E. histolytica infection had significantly higher INF-\(\gamma\) levels than uninfected controls.23 In addition, IFN-\(\gamma\) expression levels were significantly higher in asymptomatic E. histolytica infections than in asymptomatic E. dispar infections, which suggested a role of IFN-\(\gamma\) in limiting E. histolytica invasion.24

To test the hypothesis that the capacity to produce IFN-\(\gamma\) influences susceptibility to amebiasis, we measured the ability of peripheral blood mononuclear cells (PBMCs) to produce IFN-\(\gamma\) in response to amebic antigen and correlated this with future susceptibility to invasive amebiasis. Working with our existing cohort of children in Dhaka,7–11 we report the long-term active follow-up of 209 children of known capacity to produce IFN-\(\gamma\).

MATERIALS AND METHODS

Study population. Subjects were 216 children from the ongoing cohort study on amebiasis from Mirpur, an urban slum in Dhaka, Bangladesh. Details about study subjects were previously described.7 Blood for investigating cellular immune responses was drawn from October 2001 to August 2002 when the 216 children still enrolled were 87.7 ± 0.9 months of age. Children and their parents were interviewed in their homes every other day over approximately three years (until July 2005) by health care workers, and diarrheal stools were tested for E. histolytica. In addition, surveillance stool specimens were obtained every month for detection of E. histolytica infection. During this three-year period, children continued to be followed-up until they reached both endpoints of E. histolytica infection and diarrhea. All enrolled children and their family members received free primary health care services, including medications, from the project office in Mirpur. Episodes of diarrhea were treated with oral rehydration and antibiotics or anti-amebic medications as needed.

Trained research assistants took anthropometric measurements at the time of enrollment and then every four months. The anthropometric measurement closest to the time of blood draw was taken for analysis in this study. Each child was weighed in light clothes with an electronic weighing scale. The standing heights of children were measured to the nearest 0.1 cm using a locally constructed height stick. Nutritional status
was assessed by comparing the weight and height of the study children with those of National Center of Health Statistics reference population of the same age and sex with Epi-Info version 6 software (Centers for Disease Control and Prevention, Atlanta, GA). Malnourished and stunted children were defined as a weight for age Z-score (WAZ) < -2 and a height for age Z-score (HAZ) < -2, respectively.

Informed consent was obtained from the parents or guardians, and the human experimentation guidelines of the U.S. Department of Health and Human Services were followed. The study was reviewed and approved by the Institutional Review Boards of the University of Virginia and the International Centre for Diarrhoeal Disease Research, Center for Health and Population Research (Dhaka, Bangladesh).

**Diagnosis of E. histolytica infection.** Entamoeba histolytica infection was diagnosed by detection of amebic antigen in stool using the *E. histolytica* II test (TechLab, Inc., Blacksburg VA), and a strain-specific polymerase chain reaction was conducted on a subset of the stool samples to distinguish new from relapsed infections, as previously described.11 Entamoeba histolytica infection was defined as a positive test result for amebic antigen in stool. Infection could be asymptomatic or symptomatic. A new episode of *E. histolytica* infection during the period of observation was defined as a positive *E. histolytica* stool antigen and/or culture result preceding by > 2 monthly surveillance stool samples with negative results.11 Entamoeba histolytica–associated diarrhea was defined as three or more unformed stools in a 24-hour period accompanied by a new episode of *E. histolytica* infection. This definition was validated previously in this cohort by demonstrating that diarrhea was approximately five times more common in the setting of a new infection (age-adjusted odds ratio for the association of new *E. histolytica* infection with diarrhea = 4.7, 95% confidence interval = 2.9–7.6).25 The endpoint of the study was the first episode of *E. histolytica* infection and *E. histolytica*-associated diarrhea in a child after measurement of IFN-γ and IL-5 levels.

**Isolation and culture of PBMCs.** Ten milliliters of whole blood was collected from each subject and PBMCs were isolated by centrifugation over Ficoll-hypaque density gradients, according to the manufacturer’s instruction (Sigma-Aldrich, St. Louis, MO). Cells were washed with sterile Dulbecco’s phosphate-buffered saline (PBS) (Gibco-BRL, Gaithersburg, MD) and cell viability was checked by the trypan blue exclusion method. The PBMCs (4 × 10⁶/well) were cultured in 24-well flat-bottom plates (Corning-Coster, Cambridge, MA) containing RPMI 1640 medium supplemented with 10% heat-inactivated (56°C for 30 minutes) autologous serum, 100 units/mL of penicillin G, and 100 μg/mL of streptomycin sulfate and glutamine. Soluble amebic extract (SAE) (20 μg/mL) as amebic antigen was added per well and the cells were incubated at 37°C in an atmosphere of 5% CO₂ for five days. Cells were also stimulated with phytohemagglutinin (PHA) (20 μg/mL) for five days at 37°C in an atmosphere of 5% CO₂ as a positive control. Cells without antigen were also incubated in the same conditions.

**Preparation of SAE.** Two confluent T-75 flasks of *E. histolytica* HM1:IMSS trophozoites in TYI-S33 medium were harvested by centrifugation at 900 rpm for 5 minutes at 4°C. The pellet was washed by resuspension in PBS and centrifugation three times. The final cell pellet was frozen at −70°C and then thawed. The freeze-thawed lysate was centrifuged at 10,000 x g for 10 minutes at 4°C and the supernatant was incubated with a half volume of polymyxin B agarose at room temperature for one hour. The resultant absorbed supernatant was defined as SAE. The SAE was analyzed for endotoxin by the Limulus amebocyte lysate test (Cambrex BioScience, Walkersville, MD) and had < 0.1 EU of endotoxin/mL.

**Measurement of secreted cytokines.** Interleukin-5 and IFN-γ were measured using commercially available enzyme-linked immunosorbent assay reagents (Endogen, Rockford, IL). The lowest levels for detection of these cytokines were 2.5 pg/mL. The cytokine concentrations of SAE-stimulated cultures were measured above the background of unstimulated cultures after three days of culture.

**Statistical methods.** The chi-square and Fisher’s exact tests were used for categorical variables to compare proportions between groups. A restricted spline curve was used to determine that the median value of IFN-γ was the best cutting point because below the median, the risk of *E. histolytica* diarrhea linearly decreased while above the median, the risk was relatively constant. A similar spline curve was made for IL-5. Kaplan-Meier analysis was performed to estimate survival over time and the log-rank test was performed to compare survival curves. The Cox proportional hazard model was performed to estimate the relative hazards of *E. histolytica* associated diarrhea by IL-5 or IFN-γ categories. The coefficients from linear regression for association between cytokines and HAZ or WAZ were bootstrapped to have more accurate point estimates and standard errors. All P values were two-tailed.

**RESULTS**

**Population characteristics.** Of the enrolled children, 216 (75%) of 289 were still in the cohort at the time of the blood draw for cytokine analysis and of these 209 (97%) of 216 gave consent for participation in this study. One hundred nine (52.2%) of 209 children were female. At enrollment, 39 (18.7%) were 24–36 months of age, 57 (27.3%) were 37–48 months of age, and 113 (54.1%) were 49–60 months of age. Anti-lectin IgG was present at enrollment in 98 (47%) of 209 children who were malnourished at baseline and in 69 (33%) of 209 children who were stunted at baseline.

**Entamoeba histolytica–associated infections during follow-up.** The average age of children at the time of blood draw was 88 ± 0.9 months. The percentages of malnourished and stunted children at the time of blood draw were 45% (95 of 209) and 35% (74 of 209), respectively.

After blood collection for cytokine analysis, children were followed-up for an average of 970 days. One hundred twenty-five (60%) of 209 children were infected with *E. histolytica* and 31 (15%) of 209 had symptomatic *E. histolytica* diarrhea/dysentery. A total of 18% (18 of 100) of the male children and 12% (13 of 109) of the female children had *E. histolytica*-associated diarrheal illness (P = 0.21). A total of 26% (10 of 39), 11% (6 of 57), and 13% (15 of 113) were 24–36, 37–48, and 49–60 months of age, respectively, at enrollment (P = 0.09).

**Association of IFN-γ with protection from future *E. histolytica* diarrhea.** Production of IFN-γ by PBMCs in response to SAE and PHA was measured in 209 children. Fourteen children whose PBMCs did not mount a cytokine response to
PHD stimulation were excluded from further analysis. Of the 195 children included in the cytokine response analysis, 30 had *E. histolytica* diarrhea/dysentery by the end of follow-up period.

Children who produced higher than the median level of IFN-γ (median = 580 pg/mL) had longer survival without *E. histolytica* diarrhea/dysentery (log rank test *P* = 0.03) (Figure 1). The hazard ratio from Cox proportional hazard regression was 0.45 for the high IFN-γ group versus the low IFN-γ group (*P* = 0.04). This indicated that the high level of IFN-γ was associated with a reduction in the risk of *E. histolytica* diarrhea/dysentery by more than half. By month 36, 91% of the children (95% confidence interval [CI] = 86–97%) with high levels of IFN-γ did not develop *E. histolytica* diarrhea/dysentery and only 78% children (95% CI = 70–87%) with low levels of IFN-γ were free of *E. histolytica* diarrhea/dysentery. There was no statistically significant association of the HLA DQB601 allele (associated with protection from *E. histolytica* infection and diarrhea) with IFN-γ production in response to SAE.

**Lack of an association of IL-5 with protection from future *E. histolytica* infection or diarrhea.** There was no correlation of the IL-5 response of PBMCs to future susceptibility to *E. histolytica* infection or diarrhea (log-rank *P* = 0.85). By month 36, the percentages of children who remained free of *E. histolytica* diarrhea/dysentery were similar for the above and below median IL-5 (median = 254 pg/mL) groups (87% and 84%, respectively) (Figure 2).

**Effect of nutritional status on cytokine production.** A total of 46% of the children were malnourished (WAZ < −2) and 35% were stunted (HAZ < −2) at the time of blood draw. There was a positive correlation of PBMC production of IFN-γ and a negative correlation of IL-5 with HAZ and WAZ for stimulation with SAE (where malnourished or stunted children had significantly lower IFN-γ and higher IL-5 production) (Figure 3). After adjusting for HAZ, the association of IFN-γ to protection from *E. histolytica* diarrhea/dysentery remained marginally significant (*P* = 0.07) with a hazard ratio of 0.49. Therefore, the independent protective effect of

IFN-γ was still observed even when we controlled for malnutrition.

**DISCUSSION**

This is the first longitudinal study to investigate the association between *E. histolytica* associated diarrhea/dysentery and cellular immunologic function, particularly IFN-γ and IL-5. The most important finding was that IFN-γ production by PBMCs was associated with protection from future *E. histolytica* diarrhea. There was no association of IFN-γ with protection from asymptomatic luminal infection. These results suggest that cell-mediated immunity is likely to play an important role in humans in preventing *E. histolytica* from invading into the intestinal epithelium from the bowel lumen. This was consistent with previous studies demonstrating increased IFN-γ levels in patients with asymptomatic *E. histolytica* colonization compared with controls. Knowledge that the systemic production of IFN-γ is associated with immunity provides a rational framework for vaccine and adjuvant design and evaluation.

Production of IFN-γ by PBMCs in response to SAE was lower in malnourished children. Malnutrition has been shown to be associated with amebiasis. The fact that 35% of the children were stunted, combined with the association of IFN-γ with protection, may shed light on why *E. histolytica* infection had such a dramatic impact on child health in Mirpur. Malnutrition has been shown in some studies to result in decreased levels of IFN-γ production by PBMCs, e.g., in children with vitamin A deficiency and individuals with anorexia nervosa. The high level of geohelminth infection in these children could result in a vitamin A deficiency, and will be pursued by nutritional testing of the children. Earlier studies in this community have documented vitamin A deficiency: 127 (57.5%) of 221 children were vitamin A deficient (serum retinol level ≤ 0.70 μmol/L), of which 15 (6.8%) were severely deficient (< 0.35 μmol/L) (Haque R and others, unpublished data). A direct link of starvation to immunity has
been made in mice, with the hormone leptin shown to modulate T cell function and reverse starvation-induced immunosuppression.\textsuperscript{30,31} This could also explain in part the decrease in IFN-\textgamma/ H9253 and increase in IL-5 observed in the malnourished children in Mirpur. Levels of IFN-\gamma were not associated with the HLA DQB1*0601 and DRB1*1501 class II alleles associated with protection from \textit{E. histolytica} infection and diarrhea.\textsuperscript{32} This may reflect the contribution of natural killer (NK) cells\textsuperscript{33} or cytokine gene polymorphisms\textsuperscript{34} to observed levels of IFN-\gamma produced. Supporting the importance of NK cells was our observation that only part of the IFN-\gamma produced appeared derived from the acquired immune response of the children. Interleukin-5 stimulates mast cells and eosinophils, and with transforming growth factor-\beta cooperatively stimulates IgA isotype switching of antigen-stimulated B cells at mucosal sites. Although immunity to amebiasis is not believed to require mast cells or eosinophils, immunity is associated with mucosal IgA antibody. Therefore, it was plausible that IL-5 production would correlate with protection from amebiasis.

We observed that IL-5 was secreted from PBMCs in response to amebic antigen stimulation. However, IL-5 levels were not associated with susceptibility to either \textit{E. histolytica} infection or diarrhea/dysentery. This may be due to a poor correlation of the (measured) systemic versus mucosal production of IL-5, or a reflection that even low levels of IL-5 are sufficient for IgA switching.

A limitation of the present study is that it was not possible to separate innate from acquired IFN-\gamma responses because half of the children had evidence of a prior \textit{E. histolytica} infection at the time of PBMC isolation. Another limitation of our study was the comparatively small number of children (31 of 209) with \textit{E. histolytica}-associated diarrhea/dysentery during the average follow-up period of three years. A final limitation is that the only cytokines measured were IFN-\gamma and IL-5, which prevented a fuller description of the cytokine and chemokine responses that correlate with immunity. We are planning a prospective cohort study in Mirpur where children will be entered at birth to attempt to address all of these issues.

In conclusion, our study provides evidence to support earlier \textit{in vitro} and animal model data that IFN-\gamma is important in cell-mediated immunity against amebiasis. Future work will expand the description of cellular immune functions associated with the immune state and delineate contributions of the acquired and innate immune systems. Ultimately, understanding the immune correlates of protection from amebiasis should lead to new approaches to its prevention and/or treatment.

Received September 13, 2006. Accepted for publication October 17, 2006.

Acknowledgments: We thank the parents and children of Mirpur for their participation in the study.

Financial support: This study was conducted at the International Centre for Diarrhoeal Disease Research, Center for Health and Population Research and supported by a grant (AI-43596) from the National Institutes of Health. This research was also supported in part by the Intramural Research Program of the National Human Genome Research Institute, National Institutes of Health. Rashidul Haque is a Howard Hughes Medical Institute International Research Scholar.

Disclosure: William A. Petri Jr. received research support from TechLab, Inc. and royalties from a patent license agreement with TechLab for a diagnostic test for amebiasis. These royalties accrue to the American Society of Tropical Medicine and Hygiene without benefit to William A. Petri Jr.
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