CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, Jan. 1998, p. 87–90 Vol. 5, No. 1
1071-412X/98/$04.00+0

HEp-2 Cell-Adherent *Escherichia coli* and Intestinal Secretory Immune Response to Human Immunodeficiency Virus (HIV) in Outpatients with HIV-Associated Diarrhea

JOHN J. MATHEWSON,[1]* BASSAM M. SALAMEH,[1] HERBERT L. DUPONT,[1,2] ZHI D. JIANG,[1] ANDREW C. NELSON,[1] ROBERTO ARDUINO,[1] MELINDA A. SMITH,[1] AND NICHOLAS MASOZERA[1]

Center for Infectious Diseases, The University of Texas School of Public Health and Medical School,[1] and Department of Internal Medicine, St. Luke's Episcopal Hospital,[2] Houston, Texas

Received 21 April 1997/Returned for modification 25 June 1997/Accepted 29 October 1997

HEp-2 cell-adherent *Escherichia coli* and the human immunodeficiency virus (HIV) itself have recently been incriminated as causes of chronic HIV-associated diarrhea. This study sought to determine the prevalence of these two agents among HIV-infected patients with diarrhea in an outpatient setting in the United States and to compare their prevalence to that of other commonly recognized enteropathogens known to be present in this population. HEp-2 cell-adherent *E. coli* was found in 20 of 83 (24.1%) patients with diarrhea. A diffuse pattern of adherence was the most common, found in 14 of 20 (70%) patients, followed by a localized adherence pattern (6 of 20; 30%). An intestinal secretory immune response against the p24 antigen of HIV was found in 9 of 34 (27.5%) patients with HIV-associated diarrhea. The following pathogens or products were also detected in lower frequencies: *Cryptosporidium* spp. (10.8%), *Clostridium difficile* toxin (8.8%), microsporidia (6%), *Isospora belli* (3.6%), *Blastocystis hominis* (2.4%), *Giardia* spp. (1.2%), *Salmonella* spp. (1.2%), and *Mycobacterium* spp. (1.2%). The role of HEp-2 cell-adherent *E. coli* and HIV enteric infections in patients with HIV-associated diarrhea deserves further study.

Diarrhea is a common complaint among patients infected with the human immunodeficiency virus (HIV), particularly in those with AIDS. Between 30 and 60% of HIV-infected patients have diarrhea severe enough to require medical attention at some time during the course of infection (1). In developing countries, diarrhea is even more common, occurring in 60 to 90% of HIV-infected persons (3). Much of this diarrhea among HIV-infected patients is chronic, lasting weeks or months. In many cases, it is associated with profound weight loss.

The etiology of AIDS-associated diarrhea is complex, involving both microbial and host factors. All of the traditionally recognized enteropathogens have been identified in HIV-infected patients (1). In addition to these agents, there is a large and growing group of established enteropathogens and potential enteropathogens (e.g., *Cyclospora* spp. and microsporidia) that appear to be uniquely associated with immunocompromised hosts. Even including this expanded group of potential agents in patients with HIV-associated diarrhea, a potential etiologic agent is not found in the majority of cases of HIV-associated diarrhea (1).

Recently two agents, HEp-2 cell-adherent *Escherichia coli* (12) and HIV (11), have been associated with enteropathy in AIDS-associated diarrhea. HEp-2 cell-adherent *E. coli* has been identified in patients with HIV-associated acute and chronic diarrhea in the United States (6) and south-central Africa (12). We have shown that this adherent *E. coli* occurs commonly in HIV-infected patients with chronic diarrhea (79%) and significantly more often among these patients than among HIV-negative adults with diarrhea ($P < 0.002$) in Zambia (12).

Immunologic evidence to suggest that the human immune deficiency virus may infect the gut, producing diarrhea, has been provided (11). We have previously demonstrated that an intestinal secretory immunoglobulin A (sIgA) response against HIV p24 antigen occurs significantly more often among HIV-positive Zambian adult patients with chronic diarrhea than among HIV-infected patients without diarrhea ($P < 0.001$) (11). HIV has also been detected in 30 to 70% of intestinal biopsy samples from HIV-infected patients (5).

In the present study, we sought to determine the frequency of HEp-2 cell-adherent *E. coli* and intestinal sIgA response to the p24 antigen of HIV among an outpatient population of HIV-infected patients presenting to a county clinic in Houston, Tex. We also wanted to compare the frequency of occurrence of these agents to rates of infection by better-recognized enteropathogens in this setting.

MATERIALS AND METHODS

Study population. Informed consent was obtained from all patients. This study was approved by the Committee for the Protection of Human Subjects of the University of Texas and the Institutional Review Board of the Harris County Hospital District. Stool specimens were collected from all consenting HIV-positive patients attending the Harris County (Houston) Hospital District outpatient clinic who complained of diarrhea between 30 July 1992 and 21 January 1993. Acute diarrhea was defined as passage of any number of unformed stools daily for <7 days, and persistent diarrhea was defined as passage of any number of unformed stools daily for >14 days. The patients were also asked to complete a short questionnaire concerning symptoms and history of their diarrheal disease. The most recently obtained CD4 count within 3 months and a history of receipt of antimicrobial therapy within the 2 weeks prior to stool collection were obtained from patient records, when available. Eighty-three patients furnished stool specimens. Stool specimens were placed in transport media (Meridian Diagnostics Inc., Cincinnati, Ohio) and brought to the laboratory for processing within 24 h. Stool specimens from 34 of these patients were stored at −70°C and were available for later sIgA studies. *E. coli* organisms cultured from stools were studied for HEp-2 cell adherence and for enterotoxin production by methods described below.

* Corresponding author. Mailing address: Center for Infectious Diseases, The University of Texas Medical School and School of Public Health, 1200 Herman Pressler, Houston, TX 77030. Phone: (713) 500-9371. Fax: (713) 500-9364. E-mail: JMATHEWSON@UTSPH.SPH.UTH.TMC.EDU.

HEp-2 cell adherence assay. Three *E. coli* strains per patient were tested for HEp-2 cell adherence by a previously described tissue culture assay (15). Briefly, bacterial strains were grown overnight at 35°C in Trypticase soy broth with 1% D-mannose. HEp-2 cells were grown in minimal essential medium with antibiotics on glass coverslips in 24-well sterile tissue culture plates in 5% CO_2 at 35°C until near confluence. The tissue culture cells were then washed, and minimal essential medium containing 1% D-mannose was added. Twenty-five microliters of bacterial suspension was added to each well and incubated at 35°C for 3 h. The coverslips were washed three times in phosphate-buffered saline (PBS), fixed in methanol, and stained with Giemsa. The coverslips were then mounted on slides and examined at ×1,000 with a light microscope.

sIgA against HIV p24 antigen. sIgA against HIV p24 antigen was sought from stool specimens by using an enzyme-linked immunosorbent assay, as previously described (11). Briefly, stool specimens were thawed at 25°C. sIgA was then extracted with Genetron (1,1,2-trichloro-1,2,2-trifluoroethane; Fisher Scientific, Houston, Tex.). This procedure consisted of making a 10% stool suspension (1 g in 9 ml of PBS with 25 μl of aprotinin). Ten milliliters of Genetron was added and the mixture was vortexed well. It was then centrifuged at 1,500 × *g* for 15 min. The aqueous phase was saved for sIgA determinations and was stored at −70°C.

sIgA specific for the HIV test antigens was detected by a direct enzyme-linked immunosorbent assay. The HIV antigen used was the core p24 antigen (DuPont Inc., Boston, Mass.). Previous studies have shown this antigen to be the dominant HIV antigen against which intestinal sIgA is elicited (11). Microtiter plates were coated with 50 μl of a 100-pg/ml solution in a 0.01 M PBS solution. Plates were incubated overnight at 25°C. Wells were then blocked with 200 μl of 5% BLOTTO in PBS for 1 h at 37°C. Wells were then washed six times with PBS–0.05% Tween 20. Fifty microliters of a 1:10 dilution of the fecal extracts in PBS was added to each well and incubated at 37°C for 2 h. Plates were washed six times, and then 50 μl of a 1:10,000 dilution in 5% BLOTTO of anti-human sIgA conjugated to peroxidase (Cappel Inc., Westchester, Pa.) was added to each well. This was incubated at 37°C for 2 h. After six washes 50 μl of 3 mg of 4-chloro-1-naphthol per ml in methanol with 0.003% H_2O_2 to visualize immune complexes was added to each well. The plate was incubated at 25°C for 15 min in the dark and 25 μl of 1 N HCl per well was then added to stop the reaction. Plates were read at 410 nm on a microtiter plate reader (Dynatech Laboratories, Chantilly, Va.). A positive sIgA response was defined as an absorbance value greater than twice that of the negative control. The negative control consisted of a pool of fecal extracts from 30 known HIV-negative individuals who had no diarrhea.

Detection of recognized enteropathogens. A portion of each stool specimen was concentrated for parasite detection by using the Para-Pak stool concentration system (Meridian Diagnostics Inc.). This system concentrates by a formalin-ethyl acetate method. Two smears were made from the concentrated specimen. These smears were stained by the modified Kinyoun acid-fast stain for cryptosporidia and *Isospora* spp. (7). The trichrome stain was used to detect other intestinal parasites, including *Giardia*, *Entamoeba*, and *Blastocystis* spp. (10). A portion of preserved unconcentrated specimen was used to prepare a smear that was stained with a modified trichrome stain for microsporidia (16). One hundred fields on all smears were examined at ×1,000 for parasites.

Traditional bacterial enteropathogens were sought by culture as described previously (10). Briefly, specimens were streaked onto the following plates: MacConkey, Tergitol 7, Hektoen enteric, *Yersinia* selective, thiosulfate citrate bile sucrose agar, and *Campylobacter* blood agar (BBL, Cockeysville, Md.). Specimens were also placed in 10 ml of Selenite broth (BBL) for enrichment of *Salmonella* and *Shigella* spp. All plates and the Selenite broth were incubated at 37°C for 18 to 24 h. The Selenite was then streaked onto MacConkey and Hektoen enteric plates, which were incubated overnight. The *Yersinia* selective medium was incubated at 25°C for 48 h, and the *Campylobacter* blood agar was incubated microaerophilically at 42°C for 48 h. Suspicious bacterial colonies were identified biochemically and confirmed by using the API20E system (Biomerieux Vitel, Inc., Hazelwood, Mo.). *Campylobacter* isolates were identified by Gram staining and the oxidase reaction. *Mycobacterium* spp. were sought by acid-fast staining of the stool specimens. *Clostridium difficile* toxin A was sought in stool specimens by using a commercially available enzyme-linked immunosorbent assay (TekLab, Inc., Blacksburg, Va.). *E. coli* strains were tested for heat-labile and heat-stable enterotoxins by hybridization with oligonucleotide probes (13). Enteropathogenic *E. coli* adherence factor was also sought by using an oligonucleotide probe (14).

RESULTS

Among 83 HIV-infected patients with diarrhea, 15% passed one to three unformed stools per day, 46% passed four to six unformed stools per day, and 39% passed more than six unformed stools per day. The majority of the diarrhea patients presenting to this outpatient clinic complained of persistent diarrhea (72 of 83; 87%) rather than acute diarrhea (11 of 83; 13%). Seventy-eight percent (54 of 69) of these patients whose clinic records were available had taken antimicrobial agents within 2 weeks before the collection of the stool specimen.

TABLE 1. Detection of enteropathogens from outpatients with HIV-associated diarrhea in Houston, Tex.

Enteropathogen	No. positive/ no. tested (%)
HEp-2 cell-adherent *E. coli*	20/83 (24.1)
Localized pattern	6/83 (7.2)
Diffuse pattern	14/83 (16.9)
Aggregative pattern	0/83 (0.0)
Intestinal sIgA to HIV	9/34 (27.5)
Cryptosporidium parvum	9/83 (10.8)
C. difficile toxin	3/34 (8.8)
Microsporidia	5/83 (6.0)
I. belli	3/83 (3.6)
Blastocystis hominis	2/83 (2.4)
Giardia lamblia	1/83 (1.2)
Salmonella sp.	1/83 (1.2)
Mycobacterium sp.	1/83 (1.2)

E. coli strains were grown from fecal samples from 52 of 83 patients (62.7%). Among the 83 subjects (Table 1), HEp-2 cell-adherent strains were found in 20 patients with diarrhea (24.1%). In 6 of the 83 patients (7.2%), an *E. coli* strain showing localized HEp-2 cell adherence was identified. In 14 of the 83 patients (16.9%), a diffusely adherent *E. coli* strain was identified. None of the adherent *E. coli* strains exhibited an aggregative pattern of adherence to HEp-2 cells. Ninety percent (18 of 20) of the patients with adherent *E. coli* strains had no other recognized enteropathogen. None of the *E. coli* strains identified in this study hybridized with the enteropathogenic *E. coli* adherence factor probe or the enterotoxigenic *E. coli* toxin probes; finding these two diarrheagenic *E. coli* strains in adults in the United States would be unusual.

Thirty-four patients provided stool specimens that could be extracted for assay of intestinal sIgA. In 9 of these patients (27.5%), an intestinal sIgA antibody response to the p24 antigen of HIV was identified. Seven of nine patients (77.7%) with an sIgA response to HIV had no other recognized enteropathogen in their stools.

In addition, Table 1 shows the prevalence of other traditionally recognized enteropathogens that were identified among this outpatient population. The most commonly identified etiologic agents were parasitic enteropathogens: *Cryptosporidium* spp. (10.8%), microsporidia (6%), and *Isospora belli* (3.6%). *C. difficile* toxin A was detected in 3 of 34 (8.8%) of these patients, and 2 of these patients were on antibacterial therapy at the time of evaluation (ciprofloxacin and metronidazole). A *Salmonella* strain was isolated from a single patient.

CD4 lymphocyte counts were available for 79 of the 83 patients. Table 2 gives the counts (number of CD4 lymphocytes per cubic millimeter) by pathogen identified for these patients with HIV-associated diarrhea. The CD4 T-lymphocyte counts from patients with parasitic agents were the lowest. Patients with enteric infections with HEp-2-adherent *E. coli* showed a wide range of CD4 counts (4 to 552; mean, 158 CD4 cells/mm^3). Patients with an intestinal immune response to HIV p24 were found to have a mean count of 182 CD4 cells/mm^3. Patients with no recognized enteropathogens showed an average CD4 count of 147/mm^3.

TABLE 2. CD4 counts of outpatients with HIV-associated diarrhea in Houston by etiologic agent

Enteropathogen (no. of patients)	Avg (range) CD4 cells/mm^3
HEp-2 cell-adherent *E. coli* (20)	158 (4–552)
Localized pattern (6)	138 (9–355)
Diffuse pattern (14)	166 (4–552)
Intestinal sIgA to HIV (9)	182 (2–589)
Salmonella sp. (1)	424
C. difficile toxin (2)	324 (18–630)
Blastocystis hominis (2)	153 (63–242)
Cryptosporidium parvum (9)	126 (7–322)
Microsporidia (4)	34 (5–87)
Giardia lamblia (1)	21
I. belli (3)	17 (7–36)
Mycobacterium sp. (1)	4
None (31)	147 (2–589)

DISCUSSION

Diarrhea and wasting are common problems for patients with HIV infection (1). More than 50% of patients in developed countries have diarrhea at some time during the course of HIV infection (1). In developing areas, diarrhea occurs in almost 100% of HIV-infected patients (3). We sought to determine the etiology of diarrhea in a group of HIV-positive patients with AIDS who were attending an outpatient clinic in Houston. A majority of patients complaining of diarrhea (87%) met the criterion for persistent diarrhea (>2 weeks duration). This might be explained by a greater likelihood of patients with prolonged or refractory illness to furnish stool specimens.

HEp-2 cell-adherent *E. coli* had previously been identified as the cause of diarrhea in travelers and infants acquiring diarrhea in developing countries (2, 8, 10). HEp-2 cell-adherent *E. coli* may be an important cause of persistent diarrhea in infants in developing regions. The pathogenicity of one HEp-2 cell-adherent *E. coli* strain isolated from a traveler to Mexico has been confirmed by volunteer challenge studies (9). More recently, studies of HIV-infected patients with prolonged diarrhea have suggested that these organisms may be important causes of the so called "slim" disease (6, 12).

In this study, the isolation rate of HEp-2 cell-adherent *E. coli* strains among HIV-infected outpatients with diarrhea was found to be common (24.1%). Most commonly, the adherent *E. coli* strains showed a diffused pattern of attachment to HEp-2 cells (14 of 20; 70%), followed by a localized pattern of attachment in the remaining 6 patients (30%). None of these adherent *E. coli* strains had an aggregative pattern of adherence to HEp-2 cells. This is a higher prevalence of adherent *E. coli* than was found by microscopy in the intestinal biopsy specimens of patients with AIDS-associated diarrhea and wasting (17%) in New York (6). HEp-2 cell adherence patterns of the strains isolated in New York were largely aggregative, with two strains exhibiting a diffuse adherence pattern. Infection with adherent *E. coli* was also found to be associated with weight loss and peripheral blood CD4 counts of <100/mm^3. We have reported previously that adherent *E. coli* strains were cultured significantly more often from stools of HIV-positive African patients (79%) with chronic diarrhea than among HIV-negative patients (17%) with chronic diarrhea in the same setting ($P < 0.002$) (12). These HEp-2 cell-adherent strains were also isolated significantly more often among HIV-infected patients with diarrhea than among asymptomatic HIV-infected controls ($P < 0.03$). These *E. coli* strains were almost equally distributed among the three recognized HEp-2 cell adherence patterns. Biopsy specimens from HIV-positive Zambian patients with chronic diarrhea revealed that 27% of patients had bacteria adhering to intestinal mucosa (12). The high prevalence of adherent *E. coli* in AIDS patients with diarrhea deserves further study. The finding also presents interesting therapeutic possibilities for this syndrome. A pressing question relates to the potential value of antimicrobial agents. If antibacterial drugs alter the natural history of infection by adherent *E. coli* and associated diarrhea, this would produce indirect evidence of their role in the pathogenesis of diarrhea in this setting.

In this study, we also found that approximately one-third of the patients with HIV-associated diarrhea had detectable intestinal sIgA antibodies to the p24 antigen of HIV. It is now documented that HIV infects the gastrointestinal tract of many patients with AIDS and enteric symptoms (5), and there has been speculation that in many of these patients, the associated enteropathy may be due solely to the virus. We have used intestinal sIgA response as an indicator of the enteropathogenicity of bacterial enteropathogens (4). Furthermore, we have demonstrated that there was significant association of intestinal sIgA response to p24 core HIV antigen with both acute and chronic diarrhea in Zambian HIV-infected patients (11). Thirty-two of 48 Zambian patients (67%) with HIV-associated diarrhea had an intestinal sIgA immune response to p24, compared with 9 of 34 (27.5%) among the group of patients in the United States in the present study. This difference could be due to a number of factors, including different stages of HIV infection or different rates of HIV-associated enteropathy in the two populations.

Among the traditional etiologic agents of diarrhea, we found that enteric parasites were the most commonly identified pathogens in the outpatient HIV-infected patients with diarrhea in the present study. Another study of etiologic agents of HIV-associated diarrhea revealed the common occurrence of parasitic agents (1). *C. difficile* toxin (8.8%) was commonly detected in diarrheal stools in the present study. *C. difficile* infection in this population probably is due to the fact that many of these patients had been given antimicrobial therapy prior to the study. From the patient records available, 54 of 69 patients (78.3%) were on antibacterial therapy. Previous or current use of antibacterial drugs may account for the low isolation rate of traditional bacterial enteropathogens and fecal *E. coli*.

Enteric infection by adherent *E. coli* and development of an intestinal sIgA immune response to HIV were found in a higher percentage of patients with HIV-associated diarrhea than were the traditionally recognized enteric pathogens. These two newly proposed agents might explain a portion of the currently undefinable diarrhea in AIDS patients. Future prospective and therapeutic studies will be necessary to further define the roles of these agents in HIV-associated diarrhea.

ACKNOWLEDGMENTS

We thank Ali Mosavi and Farid Hussain for identification of patients, collection of samples, and clinical data.

This work was funded in part by NIH grant 1RO1AI31356-02.

REFERENCES

1. **Bartlett, J. G., P. C. Belito, and C. L. Sears.** 1992. AIDS enteropathy. Clin. Infect. Dis. **15:**726–735.
2. **Bhan, M. K., V. Khoshoo, H. Sommerfelt, P. Raj, S. Sazawal, and R. Srivastava.** 1989. Enteroaggregative *Escherichia coli* and *Salmonella* associated with nondysenteric persistent diarrhea. Pediatr. Infect. Dis. J. **8:**499–502.
3. **Dallabetta, G. A., and P. G. Miotti.** 1992. Chronic diarrhea in AIDS patients

in the tropics: a review. Trop. Doct. **22:**3–9.
4. **Jiang, Z. D., A. C. Nelson, J. J. Mathewson, C. D. Ericsson, and H. L. DuPont.** 1991. Intestinal secretory immune response to infection with *Aeromonas* species and *Plesiomonas shigelloides* among students from the United States in Mexico. J. Infect. Dis. **164:**979–982.
5. **Kotler, D. P., S. Reka, A. Borcich, and W. J. Cronin.** 1991. Detection, localization, and quantitation of HIV-associated antigens in intestinal biopsies from patients with HIV. Am. J. Pathol. **139:**823–830.
6. **Kotler, D. P., T. T. Giang, M. Thiim, J. P. Nataro, E. M. Sordillo, and J. M. Orenstein.** 1995. Chronic bacterial enteropathy in patients with AIDS. J. Infect. Dis. **171:**552–558.
7. **Ma, P., and R. Soave.** 1983. Three-step examination for cryptosporidiosis in 10 homosexual men with protracted watery diarrhea. J. Infect. Dis. **147:**8248.
8. **Mathewson, J. J., P. C. Johnson, H. L. DuPont, D. R. Morgan, S. A. Thornton, L. V. Wood, and C. D. Ericsson.** 1985. A newly recognized cause of travelers' diarrhea: enteroadherent *Escherichia coli*. J. Infect. Dis. **151:**471–475.
9. **Mathewson, J. J., P. C. Johnson, H. L. DuPont, T. K. Satterwhite, and D. K. Winsor.** 1986. Pathogenicity of enteroadherent *Escherichia coli* studied in adult volunteers. J. Infect. Dis. **154:**524–527.
10. **Mathewson, J. J., R. A. Oberhelman, H. L. DuPont, F. del la Cabada, and E. V. Garibay.** 1988. Enteroadherent *Escherichia coli* as a cause of diarrhea among children in Mexico. J. Clin. Microbiol. **25:**1917–1919.
11. **Mathewson, J. J., Z. D. Jiang, H. L. DuPont, C. Chintu, N. Luo, and A. Zumla.** 1994. Intestinal secretory IgA immune response against human immunodeficiency virus among infected patients with acute and chronic diarrhea. J. Infect. Dis. **169:**614–617.
12. **Mathewson, J. J., Z. D. Jiang, A. Zumla, C. Chintu, N. Luo, S. R. Calamari, R. A. Genta, A. Steephen, P. Schwarz, and H. L. DuPont.** 1995. HEp-2 cell adherent *Escherichia coli* in patients with human immunodeficiency virus-associated diarrhea. J. Infect. Dis. **171:**1636–1639.
13. **Murray, B. E., J. J. Mathewson, H. L. DuPont, and W. E. Hill.** 1987. Utility of oligodesoxyribonucleotide probe for detecting enterotoxigenic *Escherichia coli*. J. Infect. Dis. **155:**80–81.
14. **Nataro, J. P., M. M. Baldini, J. B. Kaper, R. E. Black, N. Bravo, and M. M. Levine.** 1985. Detection of an adherence factor of enteropathogenic *Escherichia coli*. J. Infect. Dis. **152:**560–565.
15. **Vial, P. A., J. J. Mathewson, H. L. DuPont, L. Guers, and M. M. Levine.** 1990. Comparison of two assay methods for patterns of adherence to HEp-2 cells of *Escherichia coli* from patients with diarrhea. J. Clin. Microbiol. **28:**882–885.
16. **Weber, R., R. T. Bryan, R. L. Owen, C. M. Wilcox, L. Gorelkin, and G. S. Visvesvara (Enteric Opportunistic Infections Working Group).** 1992. Improved light-microscopical detection of microsporidia spores in stool and duodenal aspirates. N. Engl. J. Med. **326:**161–166.