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Enteric Virus Infections and Diarrhea in Healthy and Human Immunodeficiency Virus-Infected Children

MARY B. LISTE,[1] IVELISSE NATERA,[2] JOSÉ A. SUAREZ,[2] FLOR H. PUJOL,[1] FERDINANDO LIPRANDI,[1] AND JUAN E. LUDERT[1]*

Center for Microbiology and Cell Biology, Instituto Venezolano de Investigaciones Cientificas,[1] and Infectology Service, Hospital for Children J. M. de los Ríos,[2] Caracas, Venezuela

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Forty-three stool samples from 27 human immunodeficiency virus (HIV)-seropositive children and 38 samples from 38 HIV-negative children, collected during a 15-month period, were examined for enteric viruses. Diagnostic assays included enzyme immunoassays for rotavirus, adenovirus, and Norwalk virus; polyacrylamide gel electrophoresis for picobirnavirus and atypical rotavirus; and PCR for astrovirus and enterovirus. Specimens from HIV-positive children were more likely than those of HIV-negative children to have enterovirus (56 versus 21%; $P < 0.0002$) and astrovirus (12 versus 0%; $P < 0.02$), but not rotavirus (5 versus 8%; $P > 0.5$). No adenoviruses, picobirnaviruses, or Norwalk viruses were found. The rates of virus-associated diarrhea were similar among HIV-positive and HIV-negative children. Enteroviruses were excreted for up to 6 months in HIV-positive children; however, no evidence for prolonged excretion of poliovirus vaccine was observed. These results suggest that although infection with enterovirus and astrovirus may be frequent in HIV-infected children, enteric viruses are not associated with the diarrhea frequently suffered by these children.

It has been estimated that by the year 2000, five to ten million children will have been infected with the human immunodeficiency virus (HIV) (34). Acute and chronic cases of diarrhea are both major sources of morbidity and mortality in these infected children, particularly in developing countries (20, 28, 36), but the etiology and pathogenesis of these gastrointestinal problems are not well understood. Functional and structural intestinal abnormalities, infection with HIV itself, and multiple opportunistic infections have all been implicated as causes of diarrhea (35). *Cryptosporidium* sp. has been the most commonly described infectious pathogen in HIV-infected children with diarrhea (4, 6, 15), but enteradherence factor-positive *Escherichia coli* (28) and nonenteric viruses, such as cytomegalovirus and herpes simplex virus, have also been implicated (31). While the role of enteric viruses in causing diarrhea in HIV-infected adults has been addressed in several studies (discussed in references 13 and 14), studies of a similar nature in children are scarce (8, 16, 27). Studies in children have found increased prevalence of enteric viruses in HIV-infected children compared to noninfected children, but associated illness appears to be neither more common nor more severe in HIV-infected children (8, 16, 27). More information is needed to fully assess the role of enteric viruses, especially the novel enteric viruses, in diarrheal diseases of HIV-infected children. This knowledge may improve the management of diarrheas, particularly in developing countries, where highly active antiretroviral therapy (HAART) is not yet widely available and where opportunistic infections continue to be a problem (7).

The aim of this study was to investigate whether infections with enteric viruses play a role in the frequent diarrheas that occur in HIV-infected children, using diagnostic assays to detect both common and novel enteric viruses. In addition, because the immunocompromised patients may develop chronic enterovirus infections (37) and prolonged excretion of oral polio vaccine (OPV) (19, 23), immunodeficient children may represent a potential reservoir for polioviruses after polioviruses in the wild have been eradicated and OPV immunization has been discontinued (19). Therefore, a second purpose of this study was to evaluate the frequency of infection with the enteroviruses echovirus, coxsackievirus, and poliovirus in HIV-infected children.

MATERIALS AND METHODS

Study population. A total of 81 fecal samples were collected between July 1997 and October 1998 from 65 children less than 5 years of age. Forty-three of the stool samples were collected from 27 HIV-seropositive children (average age, 21.2 months; range 1 to 60 months). The remaining 38 samples were collected from 38 HIV-seronegative children (average age, 16.9 months; range, 5 to 35 months; $P > 0.5$ versus average age of HIV-seropositive children). The male-to-female ratio was 1:1 for both groups. All the HIV-seropositive children attended the Infectious Diseases Service at the Hospital for Children "J. M. de los Rios", Caracas, Venezuela. The children included in this study represent approximately 70% of the total HIV-positive population attending the Service during the study period. Two children had acquired HIV by horizontal transmission and the rest had acquired HIV by vertical transmission. Patients received non-HAART treatment. The Infectious Diseases Service is a national reference center for pediatric AIDS and receives children from all over the country. Fifteen of the HIV-seronegative children also attended the Infectious Diseases Service in the Hospital for Children "J. M. de los Rios". The other 23 HIV-seronegative children attended a private clinic for routine consultation, and the fecal samples were requested by the clinicians for reasons other than this study. Verbal informed consent to collect the stools was obtained from the parents or guardians of the children enrolled at the hospital.

HIV-infected children were classified according to the Centers for Disease Control and Prevention (Atlanta, Ga.) guidelines for pediatric patients. Children were also classified as with or without diarrhea irrespective of their HIV status. Diarrhea was defined as an increased frequency of bowel movements within a 24-h period, with decrease in stool consistency (watery or liquid stools). Controls were defined as children without diarrhea for at least 7 days. Samples from patients with diarrhea were collected within 72 h after the onset of symptoms.

Eleven of the HIV-seropositive patients provided more than one fecal sample to the study. To avoid collecting more than one sample per episode of diarrhea, there was a time interval of at least 3 weeks between sample collections; each sample was considered one episode.

Fecal specimens. Samples were collected in plastic boxes and stored at −20°C until processed. Fecal suspensions, 20% in phosphate-buffered saline buffer, were clarified by low-speed centrifugation.

Aliquots of the supernatants were tested directly for group A rotavirus, adenovirus hexon group antigen, and Norwalk virus by noncommercial sandwich

* Corresponding author. Mailing address: Center for Microbiology and Cell Biology, IVIC, Carretera Panamericana, km. 11, Apdo. 21827, Caracas 1020-A, Venezuela. Phone: 58-2-5041655. Fax: 58-2-5041382. E-mail: jeludert@pasteur.ivic.ve.

TABLE 1. Viral agents detected in stool samples from HIV-positive and HIV-negative children with and without acute diarrhea

Virus	Specimens					
	HIV positive (no. [%] with virus)		*P* value[a]	HIV negative (no. [%] with virus)		*P* value[a]
	Diarrhea (n = 18)	No diarrhea (n = 25)		Diarrhea (n = 17)	No diarrhea (n = 21)	
Rotavirus	2 (11)	0	0.1694	3 (18)	0	0.0806
Astrovirus	3 (17)	2 (8)	0.6344	0	0	
Adenovirus	0	0		0	0	
Norwalk virus	0	0		0	0	
Picobirnavirus	0	0		0	0	
Enterovirus	12 (67)	12 (48)	0.1320	6 (35)	2 (10)	0.1066

[a] Fisher's exact test.

enzyme immunoassays (EIA) as described previously (13, 17). Positive controls included in the EIA were tissue-culture-grown porcine rotavirus strain OSU, human fecal samples positive for adenovirus as determined by a commercial latex agglutination test (Diarlex; Orion Diagnostica, Esapoo, Finland), and Norwalk virus-like particles (kindly provided by M. K. Estes, Baylor College of Medicine, Houston, Tex.). Approximately 0.5-ml aliquots of the supernatant were extracted for nucleic acids with phenol-chloroform and ethanol precipitation. Extracted nucleic acids were analyzed by polyacrylamide gel electrophoresis and silver staining to test for atypical rotavirus and picobirnavirus (PBV) (13), and also to confirm EIA-positive results for rotavirus. Astrovirus detection was done by reverse transcription-PCR (RT-PCR) following culture of stool samples in Caco-2 cells. In brief, clarified fecal suspensions were sterilized by filtration through a 0.22-μm-pore-size filter (Millipore), treated with 5 μg of trypsin per ml, and cultured for 48 h in Caco-2 cells according to the method of Mustafa et al. (24). Cell lysates were extracted for nucleic acids with Trizol (Gibco BRL) and were analyzed for the presence of astrovirus by RT-PCR by using the astrovirus-specific primers Mon 269 and Mon 270 (26). Tissue-culture-grown astrovirus serotype 1 was tested in parallel as a positive control. Astrovirus-positive samples were confirmed and typed by direct manual nucleotide sequencing of the PCR products (T7 Sequenase version 2.0; Amersham Life Science) and comparison of the DNA sequence to prototype strains in a database (GenBank HAstV-1 U49212, -2 L13745, -3 L38505, -4 L38506, -5 U49220, -6 L38507, -7 L38508, and -8 Z66541) as described by Noel et al. (26). Sequence analysis was done using the DNAMAN 3.2 (Lynnon Biosoft) program. For enterovirus detection, nucleic acids were extracted from 0.5-ml aliquots of the fecal supernatants (13) and tested by RT-nested PCR as described by Nicholson et al. (25). To distinguish poliovirus from non-polio enterovirus, the nested PCR products were restricted with *Bst*UI and analyzed by restriction fragment length polymorphism (25). The nested PCR products were also analyzed by single-strand-conformation chain polymorphism (30). Commercial OPV (SB Biologicals, Rixemsart, Belgium) was PCR amplified in parallel and included in the assays as a positive control. Samples positive for enterovirus by RT-PCR were inoculated into Vero and rabdomyosarcoma cells for virus isolation and serotyping.

Statistical analysis. The mean age of the HIV-seropositive and HIV-seronegative populations were compared by using the chi-square test. The rates of virus detection in the specimens from both groups of patients were tested for significance with the approximation of the binomial distribution to the normal distribution test. The rates of virus detection in samples from patients with and without diarrhea, as well as the rates of virus detection in specimens from HIV patients in the different categories, were compared for significance by using the Fisher's exact test.

GenBank accession numbers. The DNA sequences uncovered in this study have been deposited in GenBank under the following accession numbers: AF211953, AF211954, AF211955 (astroviruses serotype 3), and AF211952 (astrovirus serotype 4).

RESULTS

Enteric viruses were detected in 52% (42/81) of the stool samples collected, and were found more frequently in the stools of HIV-positive children than in samples of HIV-negative children (72 versus 29%; $P < 0.0001$). Three different viruses were identified: 6% (5/81) of the samples were positive for group A rotavirus, 6% (5/81) were positive for astrovirus, and 40% (32/81) contained enterovirus. No adenovirus, Norwalk virus, atypical rotavirus, or PBV were detected. Specimens from HIV-positive children were more likely than those of HIV-negative children to have enterovirus (56 versus 21%; $P < 0.0002$) or astrovirus (12 versus 0%; $P < 0.02$), but not rotavirus (5 versus 8%; $P > 0.5$). In addition, mixed infections were only observed in samples from HIV-positive children (14 versus 0%; $P = 0.0273$). Among the HIV-negative children, no significant differences were observed in the viral detection rates between the samples collected from the Hospital and the samples collected from the private clinic (data not shown).

Of the 43 samples collected from the HIV-seropositive patients, 42% (18/43) were from patients suffering from diarrheal disease. Among the HIV-seronegative children, 45% (17/38) of the samples were from patients with diarrhea. Table 1 shows the association between the detected viruses and diarrhea. At least one viral agent was detected in 77% of the samples from HIV-positive children and in 53% of the samples from HIV-negative children with diarrhea. Rotaviruses were significantly more frequent in samples from children with diarrhea than in samples from children without diarrhea (5/35 [14%] versus 0/46; $P = 0.013$). However, the rate of rotavirus-associated diarrhea was similar among HIV-positive and HIV-negative children (11 versus 18%; $P = 0.658$). Astroviruses were not significantly associated with diarrhea in HIV-positive children and were not observed in HIV-negative children. Enteroviruses were more common in the samples from children with diarrhea than in those from children without diarrhea, for both HIV-positive and -negative children; however, these differences were not significant. The significance of the results was unchanged regardless of whether the number of patients or the number of stool samples was used as the unit of analysis.

Partial characterization of the viruses detected was attempted by using several approaches. Analysis of the rotavirus electropherotypes identified an identical electropherotype (long) among the two HIV-positive children and the two HIV-negative children from the private clinic, indicating circulation of the same rotavirus strain in both populations. A short electropherotype rotavirus was detected in the HIV-negative child from the hospital (data not shown). DNA sequence comparisons with astrovirus reference strains indicated that three of the five astrovirus isolates were serotype 3 and one was serotype 4. Two of the 32 detected enteroviruses were classified as polioviruses, and both were excreted by HIV-positive children.

Eleven of the 27 HIV-seropositive patients provided more than one sample to the study during the total follow-up time of 52.1 child months (range 1.5 to 11 months), with at least a 3-week interval between samples. Four of these patients excreted astrovirus and two excreted rotavirus, but prolonged excretion of these viruses was not observed. In contrast, six of the nine patients with enterovirus had prolonged viral excretion of up to 23.5 child months (range 1.6 to 6 months). SSCP analysis of the nested PCR products showed similar patterns for enterovirus found in separate samples from the same patient, indicating

TABLE 2. Relationship between excretion of enteric viruses and the immunological and clinical category of HIV-positive infants

$CD4^+$ T-cell category	No. of isolates in clinical categories (positive/total samples [%])				Total
	N (not symptomatic)	A (mildly symptomatic)	B (moderately symptomatic)	C (severely symptomatic)	
0 (unknown)	0	0	3/4	0	3/4 (75)
1 (25%)	0	1/3	0	0	1/3 (33)[b]
2 (15–24%)	0	0/1	0	4/4	4/5 (80)[b]
3 (<15%)	0	2/3	4/7	12/20	18/30 (60)[b]
Total		3/7 (43)[a]	7/11 (64)[a]	16/24 (67)[a]	26/42 (62)

[a] $P = 0.3971$.
[b] $P = 0.5501$.

prolonged excretion of a single enterovirus for up to 6 months (data not shown). This virus was identified as coxsackievirus A by serotypification with type-specific antisera.

To estimate the time of vaccine polio virus excretion in the HIV-infected children, information regarding oral poliovirus vaccination was obtained for 11 of the 27 HIV-seropositive children. Polioviruses were detected in only two of the children who received OPV. These children (C2 category) had both received four doses of OPV about 5 to 9 months before sample collection.

The relationship between the presence of enteric viruses and the clinical and immune category of the HIV-infected children is shown in Table 2. Patients with moderate or severe immunosuppression had a higher prevalence of viruses than those with no immunosuppression, and virus detection rates were higher in symptomatic patients than in asymptomatic patients. However, these differences did not reach statistical significance; patients in HIV clinical categories B and C showed viral detection rates comparable to those of patients in category A ($P = 0.3971$), and patients with low $CD4^+$ T-cell count did not show higher prevalence rates than those with normal $CD4^+$ T-cell counts ($P = 0.5501$).

DISCUSSION

The causes of diarrhea in HIV-infected children are not well understood. In an attempt to better understand the etiology of this syndrome, we studied the prevalence of enteric viruses in fecal samples collected from HIV-positive and HIV-negative children under 5 years of age. Our study included sensitive assays to detect common as well as novel enteric viruses. We found three different viruses in 51% of the samples collected. Viruses were more frequently detected in samples from HIV-positive children than in samples from HIV-negative children, but this increased frequency of virus excretion was not associated with diarrhea in the HIV-infected children. These findings support and expand previous studies in Italy which showed that the frequency of excretion of rotavirus and other enteric viruses was augmented in HIV-positive children, but that these infections were not associated with diarrhea in HIV-positive children more often than in HIV-negative children (8, 16). In adults infected with HIV, several studies have identified enteric viruses as important etiologic agents of diarrhea, while other studies have failed to show such an association (discussed in references 13 and 14), suggesting that the importance of enteric viruses as cause of diarrhea in HIV-infected adults varies by geographic regions. A comparable situation has not been observed in HIV-infected children. This and previous studies (8, 16, 27) indicate that enteric virus-associated cases of diarrhea are not more common in HIV-infected children than in healthy children. Furthermore, the observation that enteric viruses are more frequently found in adults with more advanced stages of HIV infection (discussed in reference 13) has not been repeated in the studies conducted in children (8, 16; this study). These discrepancies may reflect the different clinical characteristics and the generally more rapid progression of the disease in children than in adults (2, 9, 34).

Rotavirus is the most important viral agent associated with severe gastroenteritis in children (18). Taken together, the results obtained in this and previous studies (8, 16, 27) indicate that, in children, rotavirus epidemiology and its association with diarrhea do not vary significantly during HIV infection. Adenoviruses, known to be etiological agents of diarrhea in children, were not detected in this study and were not associated with disease in a previous study (8). In contrast, in HIV-infected adults, rotavirus and adenovirus infections have been associated with diarrhea in certain epidemiological settings (1, 21).

Astroviruses have been described previously as the most common viral agent associated with diarrhea in immunosuppressed adults (5, 14), but the prevalence of this group of viruses in HIV-infected children has not previously been determined. All astroviruses detected in this study were found in HIV-infected children and were equally distributed among samples from children with and without diarrhea. Frequent asymptomatic astrovirus infections have been previously observed in healthy children (12). Although astrovirus serotypes 1 and 2 are the most widely distributed worldwide (12), those detected in this study were serotypes 3 and 4. It remains to be determined whether this reflects a serotype epidemiology that is particular to Venezuela or particular to the HIV-positive population.

Although nearly 50% of Venezuelan children under 5 years of age have antibodies to Norwalk virus (28), Norwalk virus was not detected in this study, an observation that is consistent with previous results obtained in HIV-infected patients (13, 14). However, because the EIA used in this study is specific for the Norwalk virus (17), the results cannot be extrapolated to other members of the Calicivirus family.

Picobirnaviruses are novel members of the Birnavirus family, described in the feces of several species of vertebrates, including humans (22). The association of these viruses with diarrhea in animals or in healthy humans is unclear, but they have been found to be significantly associated with diarrhea in HIV-infected adults (10, 11, 14). They were not detected in this study and were not considered in similar previous studies (8, 16, 27). Clearly, additional studies are required to understand the role of PBV as opportunistic or etiological agents of diarrhea.

Enteroviruses contributed very significantly to the high frequency of virus excretion observed in HIV-infected children but were not significantly associated with diarrhea. These results are similar to those of a previous study in Italian children (16). These results indicate that enteroviruses are not likely to

be opportunistic pathogens in HIV-infected children. Excretion of enterovirus for 6 months was observed in one HIV-infected child followed for 11 months. Duration of enterovirus excretion in immunocompetent children has been observed for up to 4 to 8 weeks (37), but in immunocompromised persons, virus excretion can last for years (19, 23). This observation is particularly relevant in view of the poliovirus eradication initiative. Poliovirus was isolated from only two of the 11 children known to have received OPV vaccine, even though five were severely immunocompromised, suggesting that prolonged excretion of OPV does not occur in these children. This is an important issue that needs further confirmation.

In conclusion, enteric viruses were detected in 72% of the samples from HIV-infected patients analyzed, and although infections with enterovirus and astrovirus were more frequently found in HIV-infected children, these infections were not significantly associated with diarrhea. Furthermore, the incidence of rotavirus infections does not appear to be modified related to the course of pediatric HIV infection. Both cellular and humoral immune responses develop in most children infected with HIV (3, 32, 33), presumably conferring protection against disease caused by enteric viruses comparable to that in noninfected children. Our and others' results strongly suggest that intestinal illnesses in HIV-infected children are mainly due to factors other than infection with enteric viruses. Understanding the causes of diarrhea in HIV-infected children may permit the development of interventions to improve their quality of life.

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