**Clinical and Molecular Observations of Two Fatal Cases of Rotavirus-Associated Enteritis in Children in Italy**

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Two fatal cases of infantile rotavirus enteritis occurred in northern Italy in 2005. Both children were severely dehydrated, and death was related to severe cerebral edema. Histological examination demonstrated extensive damage of the intestinal epithelium, villous atrophy or blunting, and macrophage infiltration. The two rotavirus strains were of the G1P[8] type and the long electropherotype. The 2005 G1P[8] rotaviruses differed in the NSP4, VP3, VP4, and VP7 genes from G1P[8] rotaviruses circulating in 2004, suggesting the onset of a new G1P[8] strain in the local population.

**CASE REPORTS**

Patient 1 was a 2-year-old Caucasian boy who, on 25 April 2005, started developing diarrhea, vomiting, and a moderate fever (about 38.5°C). Over the following 12 h, the vomiting episodes gradually decreased while the diarrhea progressively worsened, reaching 8 to 10 discharges. On this basis, the young patient was dehydrated and was rehydrated per os at home by means of a solution (Humana Idravita) containing glucose (15.88 g/liter), sodium chloride (50 mmol/liter), maltodextrin (2.60 g/liter), potassium (20 mmol/liter), sodium (60 mmol/liter), and citrates (10 mmol/liter). At this stage, his general practitioner did not observe any sign of dehydration. Unfortunately, during the subsequent night, the patient's clinical picture deteriorated further, with severe hyporeactivity and asthenia. On 27 April 2005, the child was hospitalized at our pediatric emergency department in cardiorespiratory arrest. At admission, his pupils were dilated and not photoreactive; in addition, the patient had mottled extremities and labial/nail cyanosis and respiratory movements were completely absent. The child was intubated for ventilation. However, after 30 min of cardiopulmonary resuscitation, ventilatory support was discontinued and the child was pronounced dead. Permission was given for an autopsy.

Patient 2 was a 13-month-old Caucasian boy who was admitted on 29 April 2005 to our pediatric emergency department with a 24-hour history of vomiting, nonbloody diarrhea, a temperature of 40°C, and decreased oral intake. On admission, the child was in good general condition despite mild dehydration. Upon laboratory analysis, the values were as follows: ALT, 83 IU/liter; AST, 116 IU/liter; LDH, 542 IU/liter; blood glucose, 373 mg/dl. In the blood, calcium was 7.8 mg/dl while sodium, potassium, chlorine, nitrogen, and creatine were at normal levels. Blood gas analysis revealed mixed acidosis (pH 6.813; pCO2, 119.8 mm Hg; pO2, 30.9 mm Hg; HCO3, 19 mM; blood base excess, 18.1 mM). After sedation, the child was transferred to the intensive care unit but his neurological status worsened. A computer-assisted tomography scan and angiography showed diffuse cerebral edema with ischemic areas and no evidence of cerebral blood flow beyond the carotid siphon and foramen magnum from the left cerebral artery, respectively. During this time span, sodium levels were 150 to 161 mmol/liter with a chloride of 116 to 132 mEq/liter, elevated liver enzyme levels (ALT, 148 IU/liter; AST, 152 IU/liter; LDH, 1,129 IU/liter), and a peak of blood glucose (345 mg/dl). The neurological and clinical status of the child further deteriorated, and coma dépassé was established. The child was pronounced dead, and an autopsy was performed.

**Pathology findings.** Autopsies were performed in accordance with current Italian laws. At autopsy, the morphological findings on the two children were similar. In both cases, death was attributed to tonsillar herniation through the foramen magnum as a consequence of severe cerebral edema. Another
finding they had in common was a dilated bowel lumen (mainly in the ileum and jejunum) containing diffusely watery feces.

In both cases, samples were collected from all organs, fixed in a 10% buffered formalin solution for 24 h, and embedded in paraffin tissue blocks. Five-micrometer histological sections were cut and stained with hematoxylin-eosin. The histological sections were observed through a light microscope (Olympus BX 51; Olympus, Tokyo, Japan).

Upon histopathological analysis, inflammation and blood stasis were observed in the central nervous system. The small bowel wall showed multiple areas of mucosal necrosis (with disepithelization) and villous blunting, as well as lymphoplasmacytic and neutrophilic inflammatory infiltration and edema involving the lamina propria (Fig. 1A to C), whereas the intestinal wall was not altered. In the large bowel (Fig. 1D), these changes were qualitatively similar but quantitatively less marked than in the proximal intestinal districts. The mesenteric lymph nodes displayed marked reactive hyperplastic changes. The pulmonary parenchyma showed acute blood stasis with scattered endoalveolar hemorrhages and foci of agonal emphysema. In the other organs, only signs of acute blood stasis were observed.

**Virological investigations.** Only postmortem samples, including bowel contents and peripheral blood, from patient 1 were examined. For patient 2, stool and serum samples obtained during hospitalization were examined. Both the bowel contents from patient 1 and the stool sample from patient 2 revealed rotavirus-like particles upon electron microscopic observation (strain PR1598/2005/F from patient 1 and strain PR1609/2005/F from patient 2) performed as previously described (31).

Polyacrylamide gel electrophoresis (PAGE) analysis of genomic viral double-stranded RNA (dsRNA) (32) revealed typical group A rotavirus (GARV) RNA migration patterns (4-2-3-2) with a long electropherotype (e-type). In order to determine the rotavirus VP7 and VP4 genotypes, the dsRNA extracted from 10% (wt/wt) fecal suspensions in phosphate-buffered saline (pH 7.2) using the guanidine isothiocyanate-glass milk method (17), was subjected to reverse transcription-PCR (RT-PCR) using different sets of G and P type-specific primers (4, 17, 18, 20, 29). The G1P[8] Wa strain (ATCC VR-2018) was used as a reference positive control in PCRs. Amplification products were electrophoresed at 100 V for 60 min in 2% agarose gel, stained with ethidium bromide, and visualized on a UV Transilluminator.

By PCR genotyping, the two strains were characterized as G1P[8]. The RNA was extracted from peripheral blood of patient 1 and serum of patient 2 with EXTRAzol and EXTRA-
All of the 2005 G1P[8] viruses but strain PR385/2005 clustered were distinct from the 2004 viruses. In the VP4 tree, all of the revealed that the G1P[8] 2005 viruses, with a few exceptions, the 2004 strains (Fig. 2A).

All of the 2005 strains, including PR1598/2005/F and PR1609/2005/F, segregating in a subcluster clearly separated from the 2004 G1P[8] viruses (Fig. 2D).

By alignment of the deduced amino acid sequences of NSP4 genes, several mutations in the 2005 and 2004 strains were identified (data not shown) in NSP4 regions with toxigenic and functional activities. In particular, a mutation (Asn/Lys→Ser at residue 133) occurred within the amino acid (aa) 114 to 135 region, i.e., the enterotoxigenic peptide of NSP4 or diarrhea-inducing region (DIR) (3, 27, 42), and adjacent to the interspecies variable domain (ISVD) (residues 135 to 141), which appears to influence NSP4-mediated pathogenicity, NSP4 cytotoxicity, diarrhea-inducing ability, and virus virulence in vivo (3, 21, 24, 33, 34, 43, 44). Detailed analysis of the deduced amino acid sequences of the VP4 gene identified several amino acid substitutions in the 2005 and 2004 G1P[8] strains (data not shown) affecting the VP8* antigenic subunit. Conversely, no hallmark was detectable within the VP3 and VP7 deduced amino acid sequences in strains PR1598/2005/F and PR1609/2005/F compared to the other strains examined (data not shown).

To investigate whether the mutations observed affected the NSP4 and VP4 structures, secondary structure prediction was performed using the PSIPRED protein structure prediction server of the University College of London (http://bioinf.cs.ucl.ac.uk/psipred/). Three minor differences between the 2005 and 2004 viruses were mapped in the NSP4 secondary structure (Fig. 3). In the VP8* region, we mapped 6 to 11 minor structural differences (Fig. 4) between the 2005 fatal strains (PR1598/2005/F and PR1609/2005/F) and strains representative of 2004 G1P[8] rotaviruses within lineage P[8]-III (strain PR1533/2004) and lineage P[8]-I (strain PR2108/2004).

To date, the literature describing rotavirus-associated death is limited. Fatal cases in children aged <5 years were recorded in Toronto (21 cases) in the 1970s (11). In the same years, the rate of infant mortality associated with diarrhea in the United States averaged 12.8 deaths per 100,000 live births (23). Rotavirus-associated mortality has also been documented in Venezuela (21 cases) (37) and Nicaragua (52 cases) (1) among malnourished children from very poor socioeconomic classes. On the other hand, an 11-year-long diarrhea- and rotavirus-associated hospitalization and death surveillance in the United States among children reported a fatality rate of 1 death per 1,616 rotavirus-coded hospitalizations (0.06%) (14), while a Swedish 11-year surveillance of rotavirus gastroenteritis in a hospitalized pediatric population reported a fatality rate of 1 death per 984 children with rotavirus gastroenteritis (0.1%) (22).

During a 24-year surveillance for rotavirus enteritis in children hospitalized in Parma, Italy, only the two rotavirus-associated fatal cases described in this study were documented, with an overall fatality rate of 1 death per 983 rotavirus enteritis cases (0.1%; data from 1987 to 2010, 2 deaths) (M. C. Medici et al., unpublished data). These deaths occurred during an exceptional 2005 epidemic season of rotavirus enteritis (Feb-
The scale bar in each panel indicates the number of nucleotide substitutions per position. The NSP4 genogroups (A), as well as P[8] (B) and G1 in two fatal cases of rotavirus enteritis (PR1598/2005/F and PR1609/2005/F), and of group A rotaviruses from GenBank. The GenBank nucleotide contents or in stool samples. Rotavirus antigen, but not rota-
found to segregate separately. Bánkyai et al. (5) suggested that genetic differentiation into lineages may contribute to the success of G1P[8] rotaviruses in causing natural infections.

The mechanisms controlling rotavirus virulence have not been dissected and investigated in detail yet. Several genes have been implicated in these mechanisms, suggesting complex interactions of multiple genes yet under the influence of the genetic context, rather than the effect/result of a single gene (10). In this study, several amino acid substitutions were identified in the 2005 G1P[8] rotaviruses with respect to 2004 GARVs and these mutations affected the toxigenic and functional regions of NSP4 (i.e., the DIR) and the VP8* antigenic region, which can influence GARV antibody binding and the immune response. Amino acid changes in G1 VP7 neutralization epitopes and in the signal peptide motif in various lineages and sublineages detected in different rotavirus seasons are supposed to trigger the selection of variants able to escape immunity (5), representing an important survival strategy of rotaviruses.

Also, minimal differences in the conformation of NSP4 due to mutations in the diarrhea-inducing regions of NSP4, including the ISVD, can have a critical effect on enterotoxigenic activity (21). By prediction of protein secondary structure, all of the 2005 Italian G1P[8] rotaviruses were predicted to differ from the 2004 strains PR1533/2004 and PR2108/2004. Accordingly, it can be speculated that these
changes, either alone or in combination, allowed the 2005 G1P[8] strains to spread widely throughout the pediatric population during the 2005 epidemic period, as described and suggested in other studies (9). The spatial/temporal clustering of the two distinct fatal cases documented in the Parma area in 2005 seemed unusual and provided the rationale for the gathering of sequence data on these fatal rotavirus strains. The pathophysiology of rotavirus diarrhea is clearly multifactorial (38, 39). Hence, it remains unclear whether a host-related factor or inadequate initial fluid replacement was actually responsible for the deaths of the two children, rather than possible changes in rotavirus strain properties. In conclusion, the two fatal events reported in this paper demonstrate that even nowadays in industrialized countries where children rapidly receive effective attention and therapy, fatal rotavirus enteritis can occur, highlighting the need for the adoption of rotavirus immunization programs in developed nations.

Nucleotide sequence accession numbers. The GenBank accession numbers for the nucleotide sequences reported in this paper are HQ906773 to HQ906787 for the VP3 gene, HM245019 to HM245026 for the VP4 gene, HM245027 to HM245035 for the VP7 gene, and HM244999 to HM245018 for the NSP4 gene.

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