Original article

Analyses of clinical, pathological and virological features of human rotavirus strain, YO induced gastroenteritis in infant BALB/c mice

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Abstract

Experimental studies of human rotavirus infections in mice are limited and there is lack of information on the quantitative assessment of rotaviral replication and its relationship with histological changes. In the present study, consequences of human rotavirus strain, YO induced gastroenteritis in infant BALB/c mice were analyzed for the occurrence of clinical symptoms, histopathology and virological events. The infected animals developed diarrhea and dehydration and showed accumulation of vacuolated enterocytes with lodging of the rotavirus antigens and shortening of villi in the intestine over a period of 5 days. The ileum was identified as the most susceptible and supportive part of small intestine for perpetuation of rotavirus infection in mice. Rotaviral antigen/RNA in stool and RNA in intestine were detected throughout the clinical disease period. At 48\textsuperscript{e}72 h post inoculation, diarrhea was at the peak (90\textsuperscript{e}95\%) in the infected animals with increased load of viral RNA and intense pathological lesions suggesting it as the critical time point in the course of infection. The rising titers of antirotavirus neutralizing antibodies ascertained the replication of human rotavirus strain, YO in mice. These data may contribute to the understanding of pathophysiological, immunological and virological characteristics of rotavirus infections in mice.

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1. Introduction

Rotaviruses are the single most important etiologic agent of acute gastroenteritis, worldwide. These viruses primarily infect young ones of humans, animals and birds causing significant morbidity, mortality and economic loss. It has been estimated that annually over 610,000 deaths are directly attributed to rotavirus gastroenteritis among children throughout the world \cite{1}. The clinical presentation of rotavirus gastroenteritis includes watery, nonbloody diarrhea preceded by onset of vomiting and subsequent presentation of fever and dehydration.

Rotaviruses are known to replicate in the mature enterocytes near the tips of villi and alter the structure and function of the intestinal epithelium \cite{2}. Neonates of several animal species (simian, bovine, porcine, ovine, canine, lapine and murine) have been used as experimental models to define parameters of rotavirus infection, pathology, disease, immune response and test vaccines \cite{3\textsuperscript{-}12}. Among these animals, monkeys and pigs share a genetically and antigenically closer organo-physiology to human and have been described as the most appropriate models \cite{13,14}. However, experiments with such animals may be prohibitively expensive on account of costly procurement and maintenance, usually requiring gnotobiotic conditions and cesarean delivery.

To date, mouse is the most extensively used animal model to study various aspects of rotavirus infection. Numerous strains of inbred, outbred, immunologically deficient and genetically altered rotavirus naive mice are commercially available. Infection of mice <2 weeks of age with murine
rotaviruses often leads to clinical disease and intestinal pathological changes similar to that observed in human and other animal species [7]. However, the utility of murine rotaviruses in pathophysiology studies is limited by a number of factors such as fastidious nature of these viruses to grow in cell culture, attenuation during cell culture adaptation and lack of availability of wild type strains in sufficient quantities [7,15]. Oral inoculation of heterologous rotaviruses of simian, bovine and human origin is capable of causing diarrhea and pathological changes in the intestine of infant mice identical to those produced by the murine strains [16-18]. Human rotavirus strains of genotype 3 (G3) specificity have been demonstrated to be serotypically similar to murine rotaviruses [19]. Limited studies performed using human rotaviruses have consistently reported clinical disease with morphological changes in the villus epithelium of the small intestine of a suckling mouse model [17,20]. However, quantitative assessment of viral replication in the anatomical regions (duodenum, jejunum and ileum) of the small intestine and its relationship with histological changes throughout the course of infection have not been reported. The present study reports sequential clinical, histopathological and virological features and their correlation in human rotavirus strain, YO induced gastroenteritis in BALB/c mice.

2. Materials and methods

2.1. Virus and cells

The human rotavirus strain, YO with G3P [8] specificity and fetal monkey kidney derived cell line MA104 used in the study were procured from Sapporo Medical College, Sapporo, Japan and National Center for Cell Science, Pune, India respectively. The virus was propagated in MA104 cell line in the presence of trypsin. Briefly, the virus was activated with trypsin (4 μg/ml) at 37 °C for half an hour and adsorbed on the monolayers of MA104 cells with 80% cell density at 37 °C for 90 min. The cells were refed with serum free Minimum Essential Medium (MEM) containing 1 μg/ml trypsin and incubated at 37 °C until the occurrence of 4+ (100% detachment of cells) cytopathic effect (CPE). After clarification of the harvest at 10,000 rpm for 30 min, the virus suspension was centrifuged at 35,000 rpm for 2 h. ELISA End Point (EEP) titer of the stock was determined by an infectivity assay followed by ELISA [21].

2.2. Animal inoculation and clinical observations

The animal experiments were approved by Institutional Animal Ethical Committee and Institutional Biosafety Committee of National Institute of Virology, Pune, India. Conventionally bred inbred BALB/c mice were obtained from Animal House Division, NIV, Pune. The mice were tested for the presence of rotavirus antibodies by ELISA and the seronegative animals were selected for the study.

Four to five day old mice (n = 23) borne to rotavirus antibody free dams were inoculated orally with 4000 EEP of YO strain of rotavirus suspended in 50 μl phosphate buffered saline (0.01 M PBS, pH-7.4). The animals of the control group (n = 20) were fed with 50 μl of plain PBS. Prior to inoculation, the pups from different litters were randomly distributed among the dams, with seven or eight per dam to ensure maternal care and reduce confounding effect of litter size. The mice were examined individually twice daily for rotavirus induced diarrhea by gentle palpation on the abdomen. The animals were given scores from 0 to 5 based on the expression/no expression of stool (0-no fecal expression, 1-expression of brown/black formed feces, 2-soft brown feces, 3-soft yellow feces, 4-loose yellow feces, 5-liquid yellow feces) [22]. A score of ≥2 was considered as diarrhea. The outcome was expressed as percent diarrhea (defined by the number of pups with diarrhea on a daily basis), severity (defined as the sum of diarrhea scores of each pup during the course of the experiment) and duration of diarrhea (defined as time in days with diarrhea). The infected animals were also screened for weight loss during the disease period. The stool samples were collected in 100 μl of virus transport medium (VTM) and stored at −70 °C until used.

2.3. Collection and processing of tissue samples

Intestinal tissue samples were collected at 24 h intervals up to 144 hpi (hour post inoculation). At each time point three mouse pups were sacrificed from the control and rotavirus infected groups. The three anatomical parts of small intestine, duodenum, jejunum and ileum were collected and processed separately for RNA quantification and histopathology. Intestinal tissues intended for RNA quantification were homogenized and suspended in VTM (2 ml/gm) as described earlier [12]. For histopathology, the tissue segments were inflated with 10% neutral buffered formalin, treated with ascending and descending grade of alcohol and embedded in paraffin according to standard protocol [23] to prepare sections of 3–5 μm thickness.

2.4. Histopathology and immunohistochemistry assay

Sections from different parts of small intestine were stained with haematoxylin and eosin by standard procedure [23]. The intestinal pathology was evaluated by a pathologist in a semi-quantitative manner. For immunohistochemical demonstration of rotavirus antigen in the tissue sections of intestine of rotavirus infected mice, the assay was performed using polyclonal antirotavirus rabbit antibody at 1:100 dilution, with a Vector horse anti-rabbit secondary antibody (Avidin and Biotinylated horseradish peroxidase [HRP] Complex), the Vector ABC Elite label diaminobenzidine (DAB) as the choromogen (VECTASTAIN Elite ABC system, Vector Laboratories, USA) and haematoxylin as the counterstain.

2.5. ELISA

Ten percent suspension of the diarrheic stool samples were prepared in VTM and subjected to an antigen capture ELISA according to the directions of manufacturer (Rotavirus detection kit, Generic Assay, Germany).
2.6. Real time PCR

The mouse stool and intestinal tissue samples were extracted with TRIzol®LS and TRIzol® respectively (Invitrogen Life Technologies, USA) as per the manufacturer’s protocol for isolation of rotavirus RNA. Complementary DNA (cDNA) was prepared from 10 µl RNA by reverse transcription using M-MuLV reverse transcriptase (Roche, USA) in the presence of VP6 gene specific primers-VP6F-5’GACGGVGCRACTACATGGT3’ and VP6R-5’GTCCAATTCATNCCTGGTG3’ (where R = A or G; V = A, C or G; N = A,T, C or G). The resulting cDNA was used as template for real time PCR. Quantitect SYBR green PCR mastermix kit (Qiagen, USA) was used for real time PCR quantification of rotavirus VP6 RNA as described earlier[24]. Eight serial dilutions of the plasmid containing 10⁸ to 10¹ copies of rotavirus VP6 gene fragment were included in each reaction to serve as positive controls and to construct the standard curve as well as to quantitate the rotavirus in the experimental specimens. The optimal viral load cut off that can be associated with infectious intestinal disease in mice was set as per criterion described earlier[25].

2.7. In-vitro neutralization assay

To detect the antirotavirus neutralizing antibodies (NAbs) in the infected animals, the sera samples collected from the infected mice at 10 and 20 days post inoculation were tested by MA104 cell monolayer based in-vitro neutralization assay combined with a rotavirus antigen detection ELISA as described earlier [21]. The assay included constant virus concentration (100 EEP) with varying serum dilutions. The percent neutralization was calculated as 100 – [(Diln – C1)/ (C2 – C1)] × 100) wherein C1 is mean OD₄₅₀ of the cell control wells, C2 is mean OD₄₅₀ of virus dilution used in the test, Diln is mean OD₄₅₀ of mouse serum dilutions [26]. Serum sample indicating ≥50% neutralization was considered positive for the presence of neutralizing antibodies. The neutralization titers were expressed as reciprocal of the highest dilution of the serum samples indicating 50% neutralization.

3. Results

3.1. Clinical status of the mice inoculated with rotavirus

Mice inoculated with rotavirus developed diarrhea over a period of 5 days (Fig. 1). The onset of diarrhea occurred as early as 24 hpi and the highest percent diarrhea (95%) was observed at 72 hpi with increased severity of infection (mean diarrhea score 4.4). Age matched PBS inoculated control mice did not develop diarrhea at any time point. The duration of diarrhea ranged from 2 to 4 days with mean duration of 2.8 days. The attack rate of diarrheal illness among the infected animals was 100%. The animals were lethargic and had abdominal bloating with unhealthy body coats indicating dehydration. Weight loss in the infected animals during the peak disease period was minimal (p > 0.01, p = 0.144), however, the weight gain was significantly reduced as compared to those inoculated with PBS (p < 0.01) (Student t-test). Once diarrhea ceased by 144 hpi, the normal growth rate of the infected animals was resumed and became similar to that of PBS inoculated group. None of the animals died following infection.

3.2. Pathological changes in different parts of the small intestine

There were no marked gross pathological lesions except ballooning of the intestines of infected mice [Fig. 2 A(I) and (II)]. The histopathological changes in the small intestine of...
rotavirus infected mice appeared at 24 hpi and persisted through 144 hpi. The major changes included i) hydropic degeneration of the distal one third of the villi characterized by swelling of enterocytes with accumulation of large vacuoles in the cytoplasm causing displacement of nuclei and loss of polarity; and ii) diffuse necrosis of the villi tips resulting in architectural loss and villus blunting/shortening [Fig. 2B(II)]. Morphologically the affected villi became spatulated with swollen tips, tapering bases and infiltration of inflammatory cells in the villus stroma. In addition, the intestinal lumen contained mucus, while there was edematous deposition in the submucosa along with hemorrhages in a few sections. A few infected animals exhibited reduced cell density in peyer’s patches at 72 hpi as compared to PBS inoculated control animals. The histological changes were most pronounced in the villi epithelium of terminal jejunum and ileum of small intestine. Pathologically, the extent of damage of intestinal epithelium at various time points was graded as minimal (<25%), mild (25–50%) and moderate (50–75%). Accordingly, histological changes were i) minimal to mild at 24–72 hpi and minimal to no abnormality detected (NAD) at 96–144 hpi in duodenum; ii) mild to moderate at 24–72 hpi and mild to minimal to NAD at 96 to 144 hpi in jejunum; iii) mild to moderate at 24–96 hpi and mild to minimal at 120 to 144 hpi in ileum. Intensity of the pathological changes reduced 96 h onwards with decreased enterocyte vacuolation, clear lumen and increased vasculature in the villi. The regeneration of the mucosa was obvious at the villi bases with appearance of clumps of epithelial cells and the process was well advanced by 120 hpi and virtually completed by 144 hpi in the proximal and mid small intestine leaving minimal focal lesions in the ileum. Such observations were not made in the control mice at any time point during the experiment [Fig. 2B(I)]. Histologically the villi were well preserved with variable length and fingerlike shape. Enterocytes were polarized with localized nuclei at the base.

3.3. Viral shedding in diarrheic stool

The shedding of rotaviral antigen and RNA in diarrheic stools was highest at 24 hpi. It persisted up to 120 hpi, however, at reduced level (Fig. 3).

3.4. Distribution of rotaviral RNA in the small intestine

Rotaviral RNA was detected in all three parts, duodenum, jejunum and ileum of the small intestine at 24 hpi, while it could be detected continuously only in the ileum up to 120 hpi.
with maximum viral RNA load at 48 hpi (Fig. 4). However, the viral load in any part of the intestine did not exceed the load in the inoculum. Rotavirus RNA was not detected in any segment of the small intestine of control mice.

3.5. Immunolocalization of rotavirus antigens in the ileum

Based on the comparative analyses of histopathology and rotaviral RNA distribution in different parts of the small intestine of human rotavirus infected mice, ileum was found to be the most affected part. Therefore, immunohistochemistry staining on the tissue sections of ileum collected at 72 hpi was performed. The localization of rotavirus antigens was demonstrated in both enterocytes and crypt cells of the infected villi of ileum (Fig. 5B). The ileum of control mice showed lack of peroxidase staining indicating absence of rotavirus antigen (Fig. 5A).

3.6. Detection and titration of antirotavirus NAbs in the infected mice

All mice (n = 23) inoculated with rotavirus showed sero-positive to NAbs. The titers ranged from 1:200 to 1:400 at 10 day post infection and increased up to 1:1600 to 1:3200 after 20 day post infection while the uninfected control animals remained free of rotavirus antibody.

4. Discussion

The present study reports characterization of human rotavirus strain, YO induced gastroenteritis in a mouse model. In heterologous rotavirus infections, the initial load of viral inoculum, age of the animals and host genetics are of particular importance for induction of viral replication, intestinal damage and diarrhea [6,27,28]. In view of this, 4–5 day old rotavirus antibody free BALB/c mice and a higher dose of viral inoculum were employed in the study. The mice were highly susceptible to oral infection with human rotavirus strain, YO with an attack rate of 100%. The infection produced a self limiting, acute enteric disease manifesting diarrhea, dehydration and transient growth retardation in the pups. Diarrhea prevailed over a period of 120 hpi, starting after 24 h, mounting to peak at 72 h followed by decreased
severity and final resolution of the symptoms in the succeeding period. The reduced weight gain at the peak phase of clinical disease was rapidly overcome by the animals once the diarrhea ended. These findings are similar to those described as self limiting for natural rotavirus infections in human and experimental infections in different species of animals [3,17,29–33].

The histopathological changes observed in the small intestine of mice examined in the present study appeared mild to moderate following human rotavirus strain, YO infection. Consistent with the findings reported previously [17,20,34–36], vacuolar degeneration of the enterocytes leading to necrosis of villi’s tips and blunting of villi were observed as the most conspicuous and constant pathological features in the small intestine during the course of infection in mice. Necrosis, proposed as one of the causes of enterocyte death and blunting of villi in rotavirus infection have been demonstrated possibly to play a role in intestinal malabsorption causing diarrhea and weight loss [10,37,38].

In addition to vacuolation of enterocytes and villus blunting, there were number of other pathological changes such as the constriction of villus bases with swollen tips, mucus in the intestinal lumen, edema and occasional hemorrhages in the submucosa, inflammatory infiltration in the villus stroma and depletion of cell density in peyer’s patches that have not been related with enterocyte infection so far. The systemic reactions that are induced by alterations in the intestinal microenvironment due to rotavirus infection might account for these changes. Snodgrass et al. [3] have described swollen and spatulated appearance of villi together with plugging of submucosal capillaries by neutrophils in lambs following rotavirus infection. Similar evidence favoring such systemic responses has been also provided in localized calicivirus infection in calves [39].

It is to be noted that most of the injured villi gradually returned to normal 96 hpi onwards, with reduction in vacuolization, disappearance of systemic reactions and increased circulation of blood in villi epithelium. Regeneration of the intestinal mucosa was nearly complete by 144 hpi when diarrhea was also not detected in the infected animals. Thus, the recovery from rotavirus disease occurred in parallel with regeneration of damaged intestinal mucosa. These observations corroborate earlier reports describing marked but reversible structural changes in natural and experimental rotavirus infections [2,33,35,40].

The relationship between histopathology and the presence or extent of diarrhea has been described occasionally. Studies performed on intestinal biopsies from children with rotavirus gastroenteritis have shown least changes in the intestinal histology while those carried out in rotavirus infected rabbits described typical histological changes in the intestine in the absence of diarrhea [9,41]. In several animal species, profuse diarrhea was reported to occur prior to the detection of histological changes in the intestine [42–44]. In the present study, infected animals showed intense intestinal lesions along with marked diarrhea at 48–72 hpi indicating a positive relation between the parameters. Thus, the differences in species under study, virus strains, inoculated dose and other experimental conditions may be important in variable presentation of histological changes in the intestine and its relationship with diarrhea.

Stool shedding of viral antigen/RNA reflects the replication efficacies of the rotavirus strains tested in a particular host species. As evidenced by the lack of virus excretion above input titers, non murine rotaviruses have been shown to undergo limited cycles of replication in mice [6,16,18]. In the present study, the viral load in the stool and intestinal tissues of mice did not exceed the inoculated dose of virus. However, a rate of rotavirus replication appeared to be maintained to enable the virus/its RNA shedding in the stool and persistence in the intestinal tissues during the clinical disease period i.e. from 24 through 120 hpi. Thus, the viral replication in the
intestine coincided with the onset of diarrhea and its perpetuation. This observation is in agreement with an earlier study reporting shedding of human rotavirus antigen and infectious virus for 5–8 days in 5 day old rats [10], however, was in contrast with detection of human rotavirus strain (MET) of G3 specificity only up to 2 days post inoculation in whole intestinal tissue of the 7 day old Swiss mice [17]. The discrepancy between the findings may be related to differential replication efficiencies of rotavirus strains in different hosts utilized in the studies. It may also be noted that rotavirus replication in the present study was supported by immunolocalization of virus specific antigens in the infected villi epithelium at 72 hpi and elevated antirotavirus NAb response.

Various parts of the small intestine have been described to possess differential susceptibility to rotavirus infection [8,45–47]. This phenomenon has been reported to depend on host species and rotavirus strains [48,49]. The histopathological and quantitative PCR analyses of small intestine carried out in the present study affirm that rotavirus infects all of the three parts of the small intestine. The intensity of the histological lesions and viral RNA load in different parts of small intestine varied during the course of infection, however, preferential colonization of both was evident in the ileum. Similar observations have been reported in gnotobiotic dogs, pigs, mice and rats infected with homologous or heterologous rotaviruses [4,5,10,18,50]. The higher susceptibility of the ileum to rotavirus infection could be due to the higher concentration of rotavirus specific receptors and longer exposure time to infection.

In conclusion, the results presented in this study illustrated the clinical, pathological and virological events of human rotavirus strain, YO induced gastroenteritis in infant BALB/c mice. The time course of clinical disease paralleled the viral replication and histopathological lesions in the small intestine. The ileum appeared to be the most supportive part of small intestine for perpetuation of rotavirus infection in mice. This in-vivo model may be useful to study different aspects of rotavirus infection including evaluation of attenuation of candidate vaccines of G3 origin and antivirals.

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