DIFFERENTIAL TROPISM OF EB ROTAVIRUS (SEROTYPE 3) TO SMALL INTESTINE OF HOMOLOGOUS MURINE MODEL

S.S. KANWAR*, V. SINGH, V.K. VINAYAK+, A.K. MALIK, S.K. MEHTA, S. MEHTA

Departments of Gastroenterology and Experimental Medicine+, Postgraduate Institute of Medical Education and Research, Chandigarh 160 012, India

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Summary. – The 4 – 5 days-old NMRI strain infant mice were orally inoculated with EB rotavirus (serotype 3). The intestinal disaccharidases activity was studied separately in three segments of the small intestine i.e. duodenum, jejumum and ileum on day 1 to 7 post inoculation (p.i.). The severity of EB rotavirus infection correlated with a significant decrease of small intestinal lactase, maltase and sucrase on day 3 p.i. The level of maltase after the initial decline increased in all the three segments of small intestine of infected mice. However, the lactase activity remained suppressed for a relatively longer period in ileum of infected mice than in controls. These enzymes began to approach to normal value by day 5 p.i., but in ileum, lactase activity continued to be severely depressed even on day 7 p.i. Rotavirus was consistently detected in intestinal contents by ELISA on days 1 to 7 p.i. The infected mice showed a significant increase in rotavirus (serotype 3)-specific serum IgG and IgM antibody level during the declining (days 5 – 7 p.i.) phase of infection. Diarrhoea was noted up to day 6 p.i. The protracted suppression of the lactase activity in ileum in comparison to duodenum and jejunum showed a differential tropism of EB rotavirus (serotype 3) strain to the small intestine of homologous murine model.

Key words: EB rotavirus; intestinal disaccharidases; infant mice; humoral antibody

Introduction

Acute rotavirus infection is an important cause of infantile diarrhoea in humans and animals (Black *et al.*, 1980; Cukor and Blacklow, 1984). Amongst different animal models the murine one has been successfully used for studying a variety of aspects of rotavirus infection including pathogenesis and immunity (Little and Shadduck, 1982; Offit *et al.*, 1984; Strakey *et al.*, 1986; Bell *et al.*, 1987). Rotavirus inhabits the small intestine of the susceptible host, multiplies and invades the mature villus tip enterocytes while sparing the undifferentiated crypt cells (Strakey *et al.*, 1986). The infection generally progresses from proximal (duodenum) to distal (ileum) parts (Little and Shadduck, 1982) of the gut with virus shedding in stool for a considerable period of time. During a stage of peak virus load in the intestine, rotavirus may disseminate to the liver of the infected host (Kanwar *et al.*, 1993). The damage to the enterocytes impairs the functions of brush border enzymes which is a factor contributing to the virus pathogenesis. The previous studies showed an increase in the level of thymidine kinase and little change in the level of intestinal cAMP (Collins *et al.*, 1988). The physiological basis of rotavirus-induced diarrhoea has been studied in several animal models. In miniature swine, rotavirus infection was associated with decreased intestinal lactase content and increased lactase loss consistent with the hypothesis that malabsorption of carbohydrate resulted in an osmotic diarrhoea during rotavirus infection (Graham *et al.*, 1984). In mice, however, it was not possible to demonstrate the malabsorption of carbohydrate during rotavirus infection (Blacklow and Greenberg, 1991). Other mechanisms including crypt hyperplasia and net intestinal secretion were postulated to be responsible for the diarrhoea (Collins *et al.*, 1988). The reports regarding the alterations in the intestinal disaccharidases are conflicting (Wolf *et al.*, 1981; Collins *et al.*, 1988).

*Present address: Department of Biotechnology, G.N.D. University, Amritsar 143 005, India

To characterize the profile of intestinal disaccharidases during the course of rotavirus infection the present study was concentrated on alterations of the level of intestinal disaccharidases in animals tested individually instead of pooled enzyme preparations reported earlier (Wolf *et al., 1981; Collins et al.,* 1988).

Materials and Methods

Animals. Inbred NMRI strain mice of 4 – 5 days of age were used. The inbred colony of mice was rotavirus-free as tested by specific ELISA.

Virus. EB rotavirus (serotype 3), originally gifted by Dr. H.B. Greenberg (California), was passaged in 4 – 5 day-old infant mice up to the 6th passages (P_6). The P_6 rotavirus was purified and its ID_{50} causing diarrhoea in orally inoculated 4 – 5 day-old infant mice within day 5 p.i. was estimated in a standard way.

Preparation of virus inoculum. Virus purification from the orally infected mice was done as previously described (Kanwar *et al.*, 1993). After 6 passages, infected pups were sacrified on day 3 p.i. and their small intestines were pooled and processed for virus purification. The virus stock suspension was made and the ID_{50} was estimated. To make a placebo, the intestines of age matched (7 – 8 day-old) infant mice were processed essentially in the same manner.

Oral inoculation of infant mice. Eight litters (litter size 6 – 8 mice) of 4 – 5 day-old infant mice were divided into two groups. Group I comprised four litters of mice and all animals in this group were infected orally with 10 ID_{50} in 10 µl of virus suspension. The infants were kept separate from their respective mothers half an hour before and after virus inoculation. Group II comprised 4 litters of mice. The animals in this group were given an equal volume of placebo orally and were kept separate from the infected group.

Follow up. Four to six infants mice were sacrified from each group on day 1, 3, 5 and 7 p.i. for assay of intestinal disaccharidases systemic antibody response, presence of virus antigen and virus load in the large intestine. The heparinized blood from each mouse was separately collected by direct cardiac puncture for plasma separation. Small sections of duodenum, jejunum and ileum were preserved in 3% glutaraldehyde at 4 °C. Four to five µm thin sections of these biopsies were used for localization of virus antigen by immunoperoxidase staining as previously described (Kanwar *et al.*, 1993). The small intestine segments were individually processed for estimation of disaccharidases activities. The large intestine was homogenized in 2 ml of saline and the homogenate following clarification was used for the assay of systemic IgA, IgG and IgM antibodies by ELISA.

Estimation of intestinal disaccharidases activity. The following intestinal sections were prepared: (a) duodenum, approximately 1.5 – 2.0 cm section from pylorusduodenum junction onwards; (b) jejunum, next 3.0 – 3.5 cm section; (c) ileum, rest of the portion; 4.0 to 5.0 cm up to ileo-caecal junction. Each of the intestinal sections were separately homogenized manually in 3 ml of saline in a sterile homogenizer chilled in ice bath. The homogenate was centrifuged at 8,000 × g for 30 mins at 4 °C and the supernatant was retained for the assay of intestinal disaccharidases and proteins (Lowry *et al.*, 1951). The concentration of proteins was estimated using citrate instead of tartarate.

Estimation of virus load in large intestine of mice was done by an indirect ELISA. The ELISA plate (Nunc) coated with 1:500 dilution of rabbit anti-EB rotavirus hyperimmune serum was blocked with 3% bovine serum albumin and tenfold serial dilutions (from 1:20 to 10,240) of faecal samples were added to the wells, Rabbit anti-human rotavirus - Horse raddish peroxidase (HRP) conjugate (Dakkopats, Denmark) was used as a capture antibody and the reaction was developed with 1.0 mg/ml of o-phenylene diamine in phosphate-citrate buffer pH 5.2 containing 0.15% (v/v) H_2O_2. The colour reaction was developed for 15 mins at 37 °C and terminated by addition of 1N H_2SO_4. The A_{492} was read. Appropriate controls were included in each assay.

Indirect ELISA for estimation of rotavirus-(serotype 3) specific plasma IgA, IgG and IgM. The heparinized blood from the mice was individually collected in glass capillaries by direct cardiac puncture and the plasma was used for the estimation of rotavirus-specific systemic antibody response by ELISA. A crude protein-nucleoprotein preparation of SA-11 rotavirus (serotype 3) (grown in MA-104 rhesus monkey kidney cell line) at a concentration of 3 µg/ml of carbonate-bicarbonate buffer pH 9.6 was used to coat the ELISA plate. After blocking of non-specific binding sites with 3% BSA, the 1:50 dilution of individual plasma sample was added to the wells. The goat antimouse IgA or IgG or IgM antibody conjugated to HRP was used as the detector antibody. Further steps were the same as described above.

Transmission electron microscopy. The small intestinal biopsies of randomly selected infected mice were taken on day 3 p.i. in 3% glutaraldehyde pH 7.3. Post-fixation of the tissues was done in phosphate buffered 1% osmium tetraoxide for 2 hrs followed by washing in PBS and dehydration in ethanol. Embedding of the tissue was subsequently done in Epon mixture-propylene oxide. Ultrathin sections were finally stained with uranyl acetate and lead citrate. The stained preparations were viewed in Philips-201 transmission electron microscope.

Statistical analysis. Student's t-test was used to evaluate the significance of the experimental data.

Results

EB rotavirus established itself in the small intestine of 4 – 5 day-old orally infected mice within 24 hrs of inoculation as observed by the excretion profile of virus antigen in the colon contents (Fig. 1). The virus shedding increased significantly on day 3 p.i. indicating a rapid phase of virus multiplication in the gut. On days 3 – 4 p.i. most of the infant mice developed yellow watery diarrhoea which was preceded by haunchback (curled up posture), lethargy and distention of stomach. The diarrhoea resulted in a severe dehydration which was reflected by a significant (30 – 40%) body weight loss as compared to the age-matched placebo mice

($p < 0.001$). The diarrhoea was self-limiting and resolved by day 6 p.i. but the infected mice remained underweight even up to day 7 p.i. as compared to the control animals.

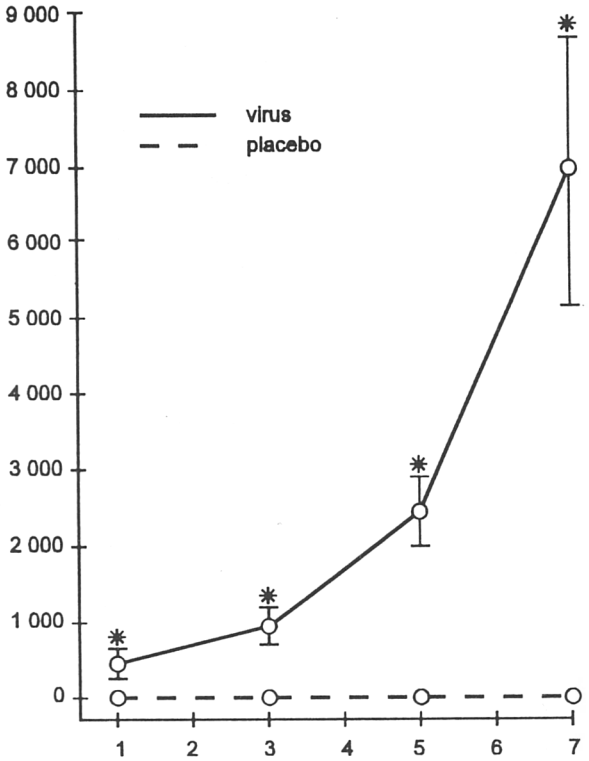

Fig. 1
EB rotavirus excretion in infant mice post infection
Abscissa: days p.i.; ordinate: virus titer (reciprocal of dilution of sample corresponding to 1 ID_{50}). Bars indicate mean standard error. *Significant at $p < 0.001$.

Disaccharidases activity in duodenum

A significant decrease in the level of lactase in duodenum on day 1 p.i. ($p < 0.001$) as compared to the control was observed which indicated more than tenfold decrease of the enzyme activity. The lactase activity in the duodenum remained significantly depressed up to day 3 p.i. (21.0 ± 4.0 units/gram protein, $p < 0.001$) as compared to the control (Fig. 2). On day 5 p.i., the lactase activity recovered and became comparable to that observed in the control but subsequently it decreased again.

Similarly, a significant decrease in maltase and sucrase ($p < 0.001$) levels was observed on days 1 – 3 p.i. (Fig. 2). The sucrase activity on day 5 increased and approached the normal value ($p < 0.05$) but on the same day the maltase activity was found to be higher ($p < 0.001$) as compared to the control. However, on day 7, virus-infected mice showed approximately fourfold and threefold elevation of maltase and sucrase activity, respectively, as compared to the controls. This increase was found to be statistically significant ($p < 0.001$).

Disaccharidases activity in jejunum

The lactase, maltase and sucrase activities in the jejunum on days 1 – 3 p.i. declined significantly ($p < 0.001$) as compared to the controls (Fig. 3). A gradual increase of the lactase activity was noticed on day 5 and it approached the normal value by day 7 p.i. The lactase activity remained significantly depressed on day 5, but the sucrase and maltase increased rapidly on day 5 p.i. This increase continued up to day 7 p.i. ($p < 0.001$) as compared to the controls.

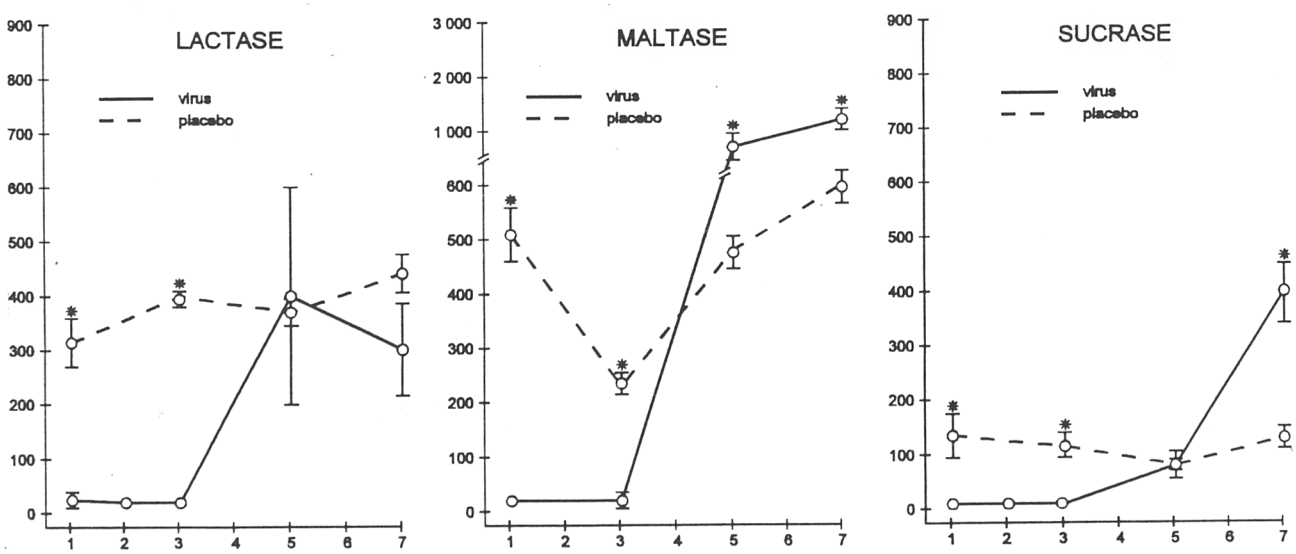

Fig. 2
Effect of EB rotavirus infection on disaccharidases activity in duodenum of mice
Ordinate: units/g protein. For the rest of legend see Fig. 1.

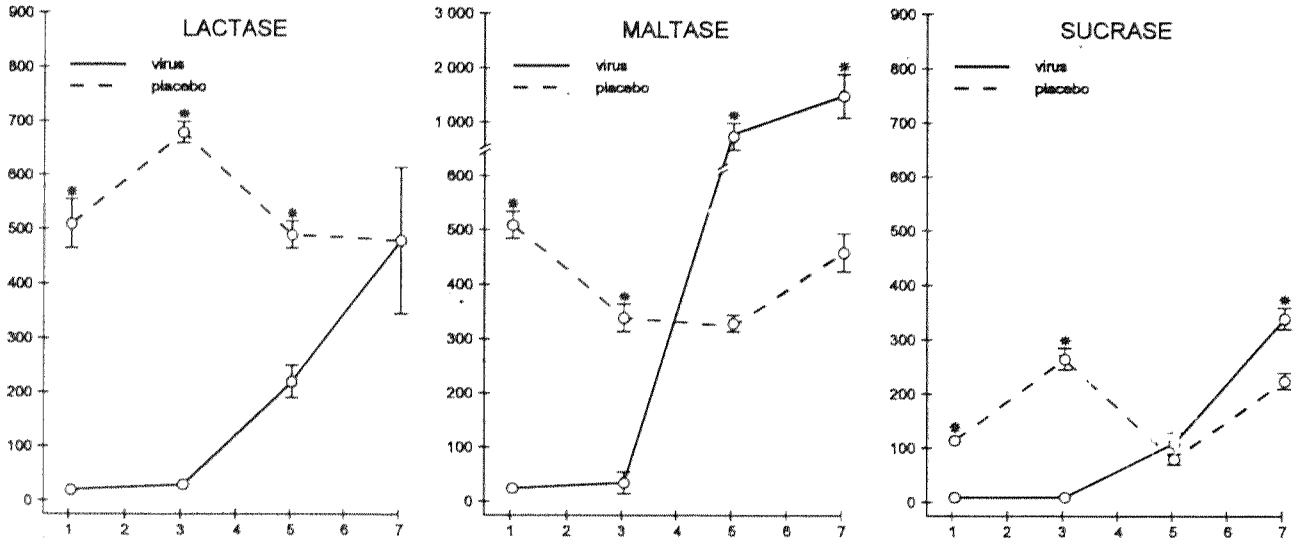

Fig. 3
Effect of EB rotavirus infection on disaccharidases activity in jejunum of mice
For legend see Figs. 1 and 2.

Disaccharidases activity in ileum

The profiles of intestinal disaccharidases in ileum of the virus-infected mice were similar to those observed in duodenum (Fig. 4). A significant decrease of lactase, maltase and sucrase was noticed on day 1 p.i. as compared to the controls. The lactase activity remained significantly depressed ($p < 0.001$) throughout the 7 days of the follow up. The maltase and sucrase activities increased gradually and attained the values comparable ($p < 0.05$) to the controls.

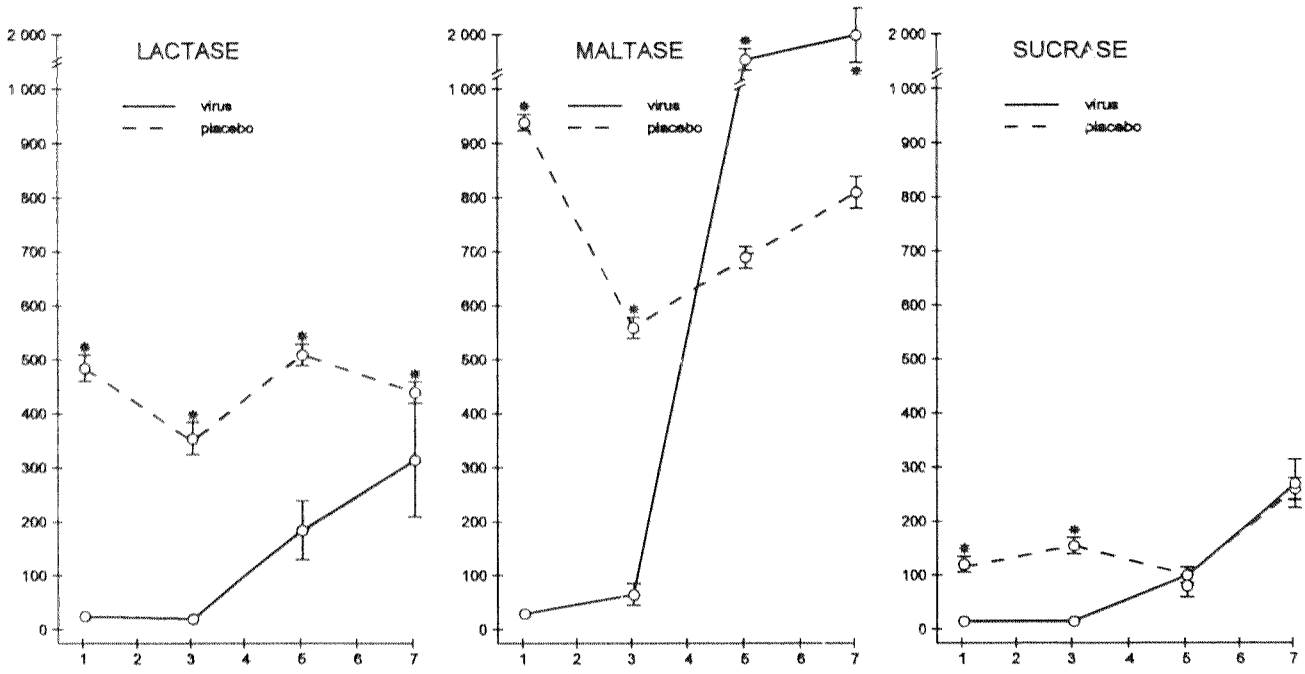

Fig. 4
Effect of EB rotavirus infection on disaccharidases activity in ileum of mice
For legend see Figs. 1 and 2.

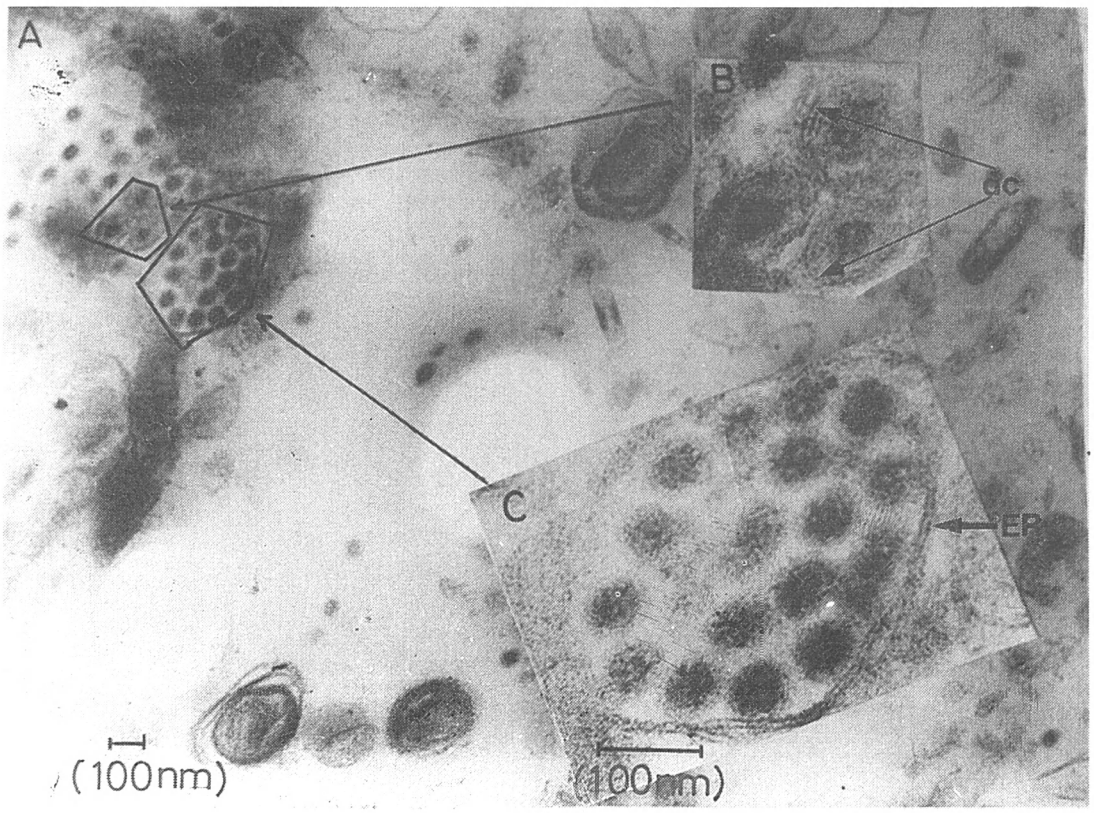

Fig. 5
Infected small intestinal biopsy
(A) single and double capsid (dc) rotavirus particles in an enterocyte; (B) complete double capsid particles at higher magnification; (C) the outer capsid was derived from the host cell endoplasmic reticulum (ER).

Virus detection in ileum biopsies

The presence of typical 70 – 75 nm in diameter, double capsid rotavirus particles in all ileal biopsies taken on days 3 – 5 p.i. from randomly selected virus-infected mice was confirmed by direct transmission electronmicroscopy. The colonies of both single and double capsid virus particles were observed in the cytoplasm of the enterocytes (Fig. 5).

Serum immunoglobulin response in virus infection

The placebo-administered animals showed a very low rotavirus-specific systemic IgG response. The specific IgA or IgM antibody response was totally absent (Fig. 6). The specific antibody response was discernible on day 3 p.i. in the infected animals. The virus-specific IgG and IgM antibody titers significantly increased ($p < 0.001$) on day 3 and this increase continued up to day 7 p.i.

Localization of virus antigen in small intestinal biopsies

The lesions inflicted by EB rotavirus in the small intestinal tissues were detected by virus-specific immunoperoxidase staining. This histopathological examination of the infected biopsies revealed presence of increased number of cell surface and cytoplasmic lesions in the enterocytes (Fig. 7A and B). However, little lesions could be discernible in the control or placebo-inoculated biopsies.

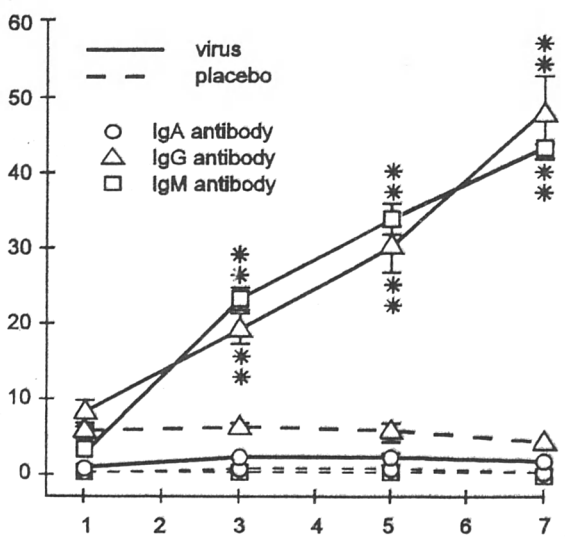

Fig. 6
Systemic EB rotavirus-specific antibody response in mice after EB rotavirus infection
Ordinate: antibody titer ($A_{492} \times 50$). For the rest of legend see Figs. 1 and 2.

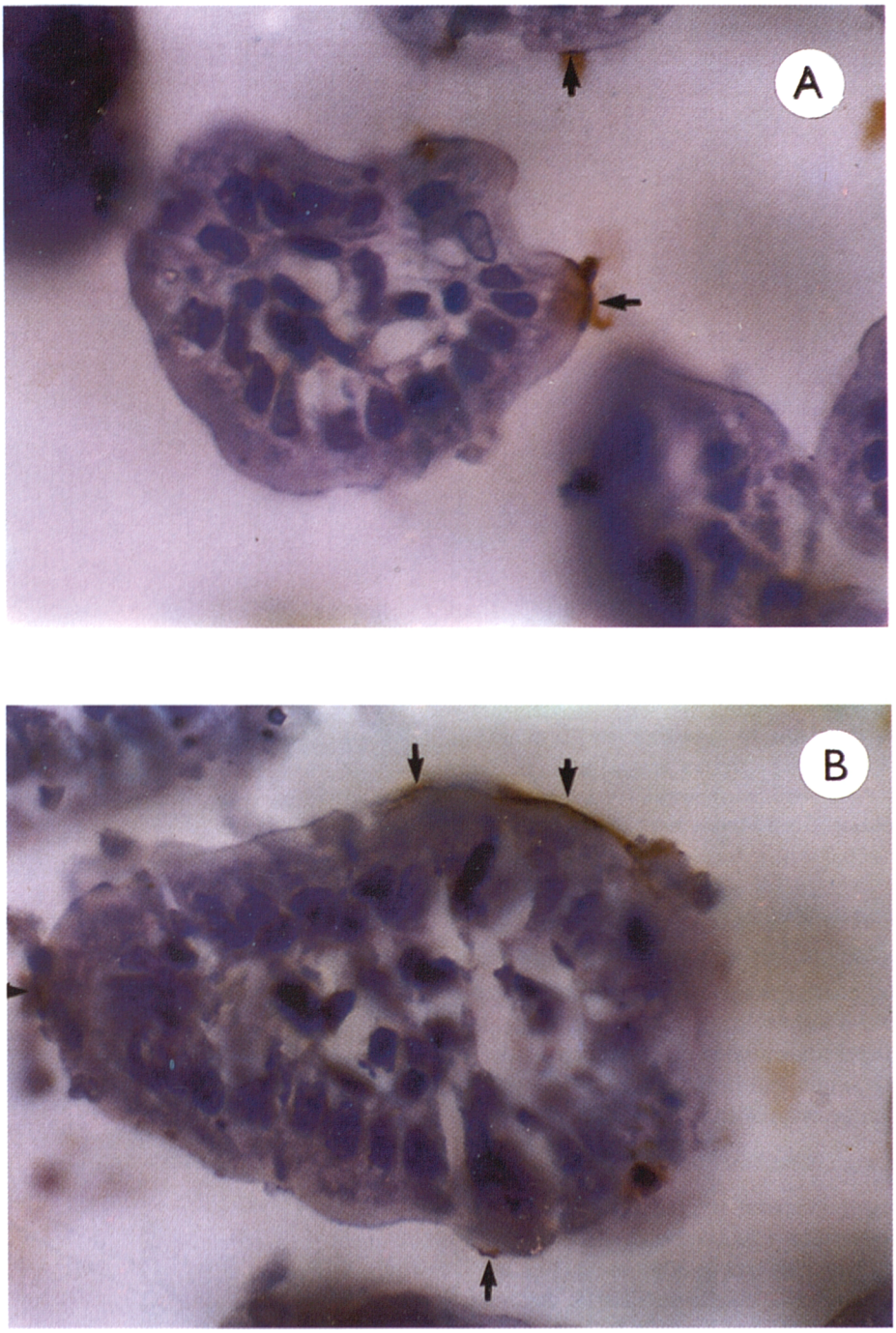

Fig. 7
EB rotavirus antigen detection by immunoperoxidase staining
Transverse section of infected mouse jejunal (A) and ileal (B) villi showing linear cell membrane positivity (brown colour marked by arrows) for virus antigen (magn. 1000 x).

Discussion

The EB rotavirus (serotype 3) infection ensued classical manifestations of gastroenteritis in infant mice. The diarrhoea was consistently observed on days 3 – 4 p.i. in the infected animals and resulted in impaired intestinal disaccharidases activities. A significant decline in disaccharidases activities on day 3 p.i. correlated well with the peak

phase of illness when the overt diarrhoeal symptoms were maximum in infected animals. The lactase malabsorption with consequent osmotic diarrhoea (Sack *et al.,* 1982) due to severe decline in intestinal lactase activity appears to be a contributing factor in the pathogenesis of diarrhoea in rotavirus infection. The enzyme profile in the ileum revealed that the infection was more protracted and severe in the ileum as compared to the proximal small intestine. This was confirmed by EB rotavirus-specific immunoperoxidase staining. The virus-infected lesions were abundant and more frequently observed in the ileal than the jejunal or duodenal biopsies. It further suggests that recovery of damaged intestinal mucosa in this infection takes longer time in ileum than in the proximal small intestine. A depression in the lactase activity has been previously demonstrated following the infection of gnotobiotic lambs and miniature swine piglets with rotavirus (Sack *et al.,* 1982).

A dramatic reduction in the lactase activity in all regions of EDIM rotavirus-infected small intestine has been noticed with minimum levels occuring on day 3 p.i. and this lactase activity didn't return to normal even on day 7 p.i. (Collins *et al.,* 1988). In the present study, though a significant decline in lactase activity was observed up to day 3 p.i. in all the three segments of small intestine, the recovery was quick in duodenum and jejunum as compared to the ileum. In a previous study, the variation in the activities of lactase, maltase and sucrase in three histologically differentiated small intestine portions (i.e. duodenum, jejunum and ileum) has been observed (Geofferey *et al.,* 1977). In human infants rotavirus infection may cause depression in one (sucrase) or more intestinal disaccharidases (Davidson and Barnes, 1979).

The prolonged impairment of the lactase activity in ileum may be due to relatively severe involvement of brush border enterocytes in rotavirus infection, because a more alkaline environment exists in the ileum than the duodenum. It may be reasoned that trypsinization (Offit *et al.,* 1986) of the rotavirus outer capsid glycoprotein VP4 is more efficient at an alkaline pH. Furthermore, the cleavage of VP4 by intestinal trypsin governs the infectivity of the rotavirus. The exact contribution of the different brush border enzymes in the rotavirus pathogenesis is not clear, but lactase malabsorption due to lactase defficiency in the rotavirus-infected host contributes to severe dehydration of the host effected by electrolyte loss. The significant depression in the intestinal disaccharidases on day 3 p.i. and later on, in rotavirus infection is also caused by replacement of infected absorptive villous cells by immature crypt cells that fail to compensate for the absorptive defect (Middleton, 1978). The systemic antibody response was not detectable until day 3 p.i., when the diarrhoeal incidence was consistently observed. The undetectable serum IgA antibody indicated that it may be the secretory IgA antibody at the site of infection which may respond to the enteric infection. The increase of the systemic IgG and IgM antibody levels indicated that these antibodies rather than the IgA may be instrumental in ceasing the rotaviral infection in the small intestine. Further studies to delineate (a) the exact role of the gut-associated immunoglobulin-secreting plasma cells, and (b) the effector functions of intraepithelial and lamina propria T-lymphocytes in rotavirus infection or immunity are in progress. Our preliminary unpublished data suggest that the plasma cells in the gut tissue are undetectable in 10 – 20 day-old mice by the specific immunoperoxidase method. It is likely that T-cell effector functions influx during the course of infection or immunization and regulate the viral enteric disease in the small-gut.

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References

Bell, L.M., Clark, H.F., Brien, E.A., Kornstein, M.J., Plotkin, S.A., and Offit, P.A. (1987): Gastroenteritis caused by human rotaviruses (serotype 3) in a suckling mouse model. *Proc. Soc. exp. Med.* **184**, 127–132.

Black, R.E., Merson, M.H., Rahman, A.S.M., Yunus, M., Alim Arma, Hug. I., Yolken, R.H., and Curlin, G.T. (1980): A two year study of bacterial, viral and parasitic agents associated with diarrhoea in rural Bangladesh. *J. infect. Dis.* **142**, 660–664.

Blacklow, N.R., and Greenberg, H.B. (1991): Viral gastroenteritis. *The New Engl. J. Med.* **325**, 252–264.

Collins, J., Starkey, W.G., Wallis, T.S., Clark, G.J., Warton, K.J., Spencer, A.J., Maddon, S.J., Osborne, M.P., Candy, D.C.A., and Stephen, J. (1988): Intestinal enzyme profiles in normal and rotavirus infected mice. *J. Pediatr. Gastroenterol. Nutr.* **7**, 264–172.

Cukor, G., and Blacklow, N.R. (1984): Human viral gastroenteritis. *Microbiol. Rev.* **48**, 157–179.

Dahlqvist, A. (1984): Assay of intestinal disaccharidases. *Scand. J. clin. Lab. Invest.* **544**, 169–172.

Davidson, G.P., and Barnes, G.L. (1979): Structural and functional abnormalities of the small intestine in infants and young children with rotavirus gastroenteritis. *Acta Pediatr. Scand.* **68**, 181–186.

Geoffrey, P., Davidson, D., Grantgall, K., and Petric, M. (1977): Human rotavirus enteritis induced in conventional piglets. *J. clin. Invest.* **60**, 1401–1409.

Graham, D.Y., Sackman, J.W., and Estes, M.K. (1984): Pathogenesis of rotavirus induced diarrhoea: preliminary studies in miniature swine piglet. *Dig. Dis. Sci.* **29**, 1028–1035.

Kanwar, S.S., Singh, V., Malik, A.K., Vinayak, V.K., Komal, H.S., Pal, S.R., Mehta, S., and Mehta, S.K. (1993): Hepatic involvement in rotavirus gastroenteritis in a homologous murine model. *Immunol. infect. Dis.* **3**, 77–81.

Little, L.M., and Shadduck, J.A. (1982): Pathogenesis of rotavirus infection in mice. *Infect. Immun.* **38**, 755–763.

Middleton, P.J. (1978): Pathogenesis of rotavirus infection. *J. Am. vet. Med. Assoc.* **173**, 544–546.

Offit, P.A., Clark, H.F., Kornstein, M.J., and Plotkin, S.A. (1984): A murine model of oral infection with a primate rotavirus (Simian SA-11). *J. Virol.* **52**, 233–236.

Offit, P.A., Blavat, G., Greenberg, H.B., and Clark, H.F. (1986): Molecular basis of rotavirus virulence: Role of gene segment 4. *J. Virol.* **57**, 46–49.

Sack, D.A., Rhodes, M., Molla, A., Molla, A.M., and Wahed, M.A. (1982): Carbohydrate malabsorption in infants with rotavirus diarrhoea. *Am. J. clin. Nutr.* **36**, 1112–1118.

Starkey, W.G., Collins, J., Wallis, T.S., Clarke, G.J., Spencer, A.J., Haddon, S.J., Osborne, M.P., Candy, D.C.A., and Stephen, J. (1986): Kinetics, tissue specificity and pathological changes in murine rotavirus infection of mice. *J. gen. Virol.* **67**, 2625–2634.

Wolf, J.L., Cukor, G., Blacklow, N.R., Dambrauskas, R., and Trier, J.S. (1981): Susceptibility of mice to rotavirus infection: Effect of age and administration of corticosteroids. *Infect. Immun.* **33**, 565–574.