Protection against rotavirus diarrhoea in mice by trypsin inhibitor

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To investigate the role of soyabean trypsin inhibitor (TI) during rotavirus (RV) diarrhoea, changes in enzyme activities of six relevant mucosal enzymes (lactase, sucrase, maltase, trehalase, glucoamylase and alkaline phosphatase) were assayed following inoculation of suckling mice with EB rotavirus (serotype 3) along with the TI and compared with the age-matched healthy control mice. The animals were divided into three groups i.e. group 1 (controls), group 2 (RV inoculated) and group 3 (RV + TI inoculated and sacrificed under light anaesthesia on 0,1,3,5, 7 and 10 day post inoculation (dpi). Then intestines were excised and divided into two parts (jejunum and ileum). They were separately homogenized in 0.9% cold normal saline and activities of mucosal enzyme were measured. Alkaline phosphatase and disaccharidases were found to be decreased significantly in RV inoculated animals in both the anatomical portions of small intestine of mice. These enzyme levels were restored with the administration of TI i.e. in group 3 and became comparable to the controls in both intestinal portions. These studies suggest that activity of intestinal enzymes which are important in digestive absorptive functions of small intestine were restored with the addition of TI when given to infant mice showing its protective efficacy during rotavirus infection.

Rotavirus infection is associated with a spectrum of disease manifestations, ranging from a sub clinical infection to severe gastroenteritis with life threatening dehydration. In developing countries, rotavirus may cause 600,000 to 870,000 deaths each year, accounting for an estimated 20 to 25% of all deaths due to diarrhoea and 6% of all deaths among children <5 years of age. In India, rotavirus is the major etiological agent in children. The pathology of rotavirus appears to be limited to the small intestinal epithelium and to the secondary effects of dehydration and electrolyte imbalance. Release of free oxygen radicals may play a role in pathogenesis of diarrhoea. Animal studies, in which the role of absorptive abnormalities in rotavirus infections was examined, have failed to identify the clear-cut mechanisms involved in the development of diarrhoea.

Till date, replacement of fluids and electrolytes is the mainstay of therapy in rotavirus gastroenteritis. Multiple reports have shown promise in vitro antiviral agents to be effective against rotavirus. Information concerning the safety, efficacy and recommended use of vaccines tried in rotavirus diarrhoea are plenty but highly controversial. Previously intestinal protease inhibitors have been reported to suppress replication of rotavirus. In our preliminary studies of trypsin inhibitor, we observed alterations in amino acid uptake and emphasis was laid on histology. We for the first time are demonstrating the protective role of trypsin inhibitor (TI) on intestinal enzymes during rotavirus diarrhoea in mice. We analysed sequentially throughout the course of rotavirus infection, the activities of five brush border enzymes important in digestive functions of the small intestine.

Materials and Methods

Inbred BALB/C mice (n=108) of 7 days age were selected for the present study and were procured from Central Animal House of Post Graduate Institute of Medical Education and Research, Chandigarh before starting the experiments. They were confirmed negative for rotavirus antibodies by ELISA as described earlier and were kept in the sterile autoclavable polypropylene cages (Tarsan, Calcutta, India). All animals were maintained on pellet diet (Hindustan Lever Ltd., Bombay India) and water ad libitum.

Murine EB rotavirus (serotype 3) used in the present studies was kindly gifted by Dr. H.B. Greenberg, Polo Alta, VA Medical Centre, Standford.
University, California, U.S.A. A stock was prepared as previously described and ID 50 titre from 9th passage (P9) was estimated by a standard method. A dose of 0.6 mg of TI/g body weight/50μl RV stock was standardized to orally inoculate each animal after a pilot study.

Animals were divided in group 1 (healthy controls) group 2 (RV inoculated) and group 3 (RV + TI inoculated) (n=36 each). Group 1 animals were orally inoculated with 50 μL normal saline, group 2 animals were orally inoculated with 50 μl RV stock (P9) each and group 3 animals were orally inoculated with 0.6 mg of TI/g body weight along with 50μl RV stock (P9) each. Sufficient time was allowed for animals to swallow the liquid. Control animals were kept separated from the infected animals along with their respective dams.

Animals were daily examined for diarrhoea by gentle palpation of abdomen and their body weight recorded on every alternate day. Six animals from each group were sacrificed under light chloroform anaesthesia on 0, 1, 3, 5, 7 and 10 days post inoculation (dpi). Whole small intestines (from pylorus to ileocecal junction) were taken out and divided into two parts. The first 1/3 was taken as jejunum and the second 2/3 was named as ileum. Small segments of both portions were kept in formalin to look for histological alterations. Intestinal portions were separately homogenized in cold 0.9% normal saline.

Disaccharidases (lactase, sucrase, maltase, glucoamylase and trehalase) activity was assayed by the method of Dahlqvist. Alkaline phosphatase activity was determined using the method of Bergmeyer. Proteins were estimated according to the method of Lowry. Student’s t-test was employed for the statistical analysis of the data and the results were expressed as mean ± standard error (SE). P-value less than 0.05 was considered as the significant value. Ethical approval from the Post Graduate Institute of Medical Education and Research, Ethical committee was obtained before starting animal experimentation. All enzyme activities were expressed as units/mg protein. One enzyme unit was equal to one μmole of substrate hydrolyzed/min under the standard assay conditions.

Results

The diarrhoeal response of mice to rotavirus and TI followed the same course as described previously. RV challenged mice developed yellowish watery diarrhoea on palpation of abdomen. With the addition of TI the severity of diarrhoea in group 3 animals decreased markedly and were normal by 7 dpi. Diarrhea in RV inoculated animals resulted in severe dehydration that was reflected by significant decrease in body weights. In RV+ TI inoculated mice, body weight was restored and were comparable to group 1 (i.e. control group) as observed in a preliminary studies.

Haematoxylin and Eosin stained sections of mouse intestine following RV inoculation showed focal areas of vacuolar degeneration of epithelial lining, mainly at the tips of the villi on the peak day of infection (3rd and 5th dpi). In RV+ TI group H & E stained sections of intestine were of normal histology.

Mucosal enzyme levels

There was a dramatic reduction in the activity of lactase in jejunum of RV infected small intestine compared to the controls. The levels were significantly reduced from 1 to 10 dpi. Similarly the levels were significantly less in ileum, however the reduction was observed from 3 dpi to 7 dpi after which they were comparable to the controls on group 3. The enzyme levels were restored on all days of the
schedule of the experiment in the jejunal section of small intestine and were comparable to the controls. In ileum a similar pattern was observed and the enzyme levels were comparable to the controls by 7 dpi (Fig. 1).

Sucrase enzyme activity was significantly decreased in jejunum of small intestine in-group 2 animals on 1, 3 and 5 dpi. A similar trend was observed in the ileal section compared to the controls. However on the addition of TI the levels were less than the controls on 1 dpi and thereafter became comparable till the end of schedule of experiment. In ileum the levels increased but not significantly compared to the group 2 and were less than the group 1 (controls) on 5 dpi. But thereafter the enzyme levels were found to be comparable to the controls (Fig. 2).

Maltase enzyme levels decreased significantly in jejunum of RV inoculated animals compared to the controls on 1, 3, and 5 dpi. In ileum, decreased enzyme activity was observed on 3 and 5 dpi in group 2 compared to the controls. When group 3 was compared to group 2, enzyme levels were found to be restored on peak days of infection and became comparable to the controls in the jejunum. The levels however remained reduced in ileum on 3 and 5th dpi and thereafter became comparable to the controls (Fig. 3).

Fig. 2 — Effect of rotavirus and trypsin inhibitor on intestinal sucrase activity in infant mice

Fig. 3 — Effect of rotavirus and trypsin inhibitor on intestinal maltase activity in infant mice

The levels of glucoamylase were decreased significantly (P<0.001) in group 2 animals in the jejunum of small intestine on 3, 5, and 7 dpi compared...
to the controls. In ileum, the levels were reduced significantly ($P<0.001$) on 5 and 7 dpi compared to the group 1 (controls). However on 3 dpi the levels were less when compared to the controls, and thereafter became comparable on all days of schedule of experiment. In ileum the enzyme levels increased though not significantly in group 3 and were comparable to the controls on all days of the schedule (Fig. 4).

Trehalase enzyme levels were reduced significantly ($P<0.001$) on 3, 5, and 7 dpi in group 2 in the jejunum compared to the group 1. In ileum, reduced enzyme levels were observed on 3 dpi in RV inoculated animals compared to the controls. In groups 3, the levels became comparable to the controls in jejunum. In ileum, enzyme levels became comparable to group 1 on all days of the experimental schedule (Fig. 5).

The alkaline phosphatase activity of jejunum was significantly ($P<0.001$) lower in group 2 animals compared to the group 1 on 3 and 5 dpi. A similar level of significance was observed in the ileum section of small intestine in group 2 compared to group 1. However the levels were less on 3 dpi ($P<0.001$) in group 3 when compared to group 1 and thereafter became comparable to the controls on all days of experimental schedule. In ileum, the levels were still lower on 3 dpi which thereafter became comparable on all days of the schedule (Fig. 6).

### Discussion

In the present studies, we show the protective role of TI, as the mucosal enzyme activities were restored and become comparable to the healthy control animals. This can be supported by the histological findings after the administration of TI as reported earlier by our lab\(^{10}\). It has been proposed that enterocytes repopulating villi after virus-induced tip cell damage could be crypt-like with a reduced digestive and absorptive capacity\(^{15}\). However, when the TI is inoculated to the animals, enterocyte, repopulating villi are differentiated resulting in normal digestive and absorptive capacity of villi.

The results of the present investigation, along with the previous pathophysiological and malnutrition studies\(^{17,18}\) suggest that the infection in suckling mice with rotavirus (serotype 3) provides a good animal model for the analysis of mechanisms resulting in rotavirus induced diarrhoea. In consistence with earlier reports of rotavirus infection in mice\(^{10,11}\) no mortality was observed.

Kinetic tissue specificity and pathological studies\(^{15}\) indicate that the rotavirus invades and replicates in enterocytes of upper portion of villi, resulting in vacuolation and degeneration in epithelium of tips of villi. This explains for the significant reduction in the activities of alkaline phosphatase and lactase. Both these enzymes were restored when TI was inoculated...
along with the RV showing its protective role in rotavirus diarrhoea. This was found to be correlated well with the histological findings on the administration of TI to the infant mice.

Mucosal enzymes can serve as markers for the small intestinal damage. Disaccharidases are localized mainly in the brush-border. Our data suggests that acute rotavirus infection lead to decreased small bowel enzyme activities (both in jejunum and ileum). In early infancy, small intestine undergoes a period of rapid growth and the cell turnover is very high. The development associated with multiple changes of intestinal structure and functions is at a very high rate so during postnatal period the infant is highly susceptible to intestinal injury. No information is available till date on the effect of TI on brush-border and the enzymes present there. Earlier we showed the protective efficacy of TI on leucine amino peptidase activity and glutathione levels in the small intestine under the stress of rotavirus infection and malnutrition.

Glucoamylase and trehalase were the two new enzymes important in generating absorbable monosaccharides estimated in the present study. To our knowledge, there have been no other reports documenting the effects of RV and TI in any other animal species and humans. The activities of these two enzymes followed the same pattern as other disaccharidases and results with TI augment the findings of other mucosal enzymes estimated in our studies.

Certainly, the results of these model studies do not eliminate the possibility that TI given during RV diarrhoea would be useful against rotavirus infections as elucidated by our studies of mucosal enzymes which were restored on the administration of TI to infant mice during rotavirus diarrhoea.

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References


