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Experimental infection of pigs with group A rotavirus and enterotoxigenic *Escherichia coli* in India: gross, histopathological and immunopathological study

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Summary

The authors describe a detailed study conducted in Assam, India, of gross, histopathological and immunopathological alterations in pigs experimentally infected with rotavirus and enterotoxigenic *Escherichia coli* (ETEC) expressing K88 pili. A total of 30 Caesarean derived piglets were infected experimentally with rotavirus alone or in combination with ETEC to study the gross and histopathological alterations and the distribution pattern of different B- and T-cell subsets in the gut. Villus atrophy, especially in the jejunum and ileum, was the consistent lesion in piglets infected with rotavirus, while in piglets simultaneously infected with rotavirus and ETEC, severe necrosis of the intestinal villi was observed. Ultrastructural studies revealed similar pathological alterations in the ileum of the infected piglets. A morphometric study of the intestinal villi and crypts showed a reduction in the ratio between the average villus height and crypt depth (VH:CD ratio) in the group infected with rotavirus (5.95 ± 0.33) and those infected with rotavirus and ETEC (7.90 ± 0.16). A higher (p<0.01) reduction in the VH:CD ratio was observed in the jejunum (8.83 ± 0.79) and ileum (8.46 ± 0.78) compared that in the duodenum (10.03 ± 0.50) of the infected pigs. Piglets infected with rotavirus and sacrificed on day 6 post infection revealed the presence of lymphocytes containing cytoplasmic IgA^+ ($cIgA^+$) cells in the villus lamina propria and intra-epithelial $CD8^+$ T-cells in the villus epithelia. Rotavirus infection of young piglets in association with ETEC was more severe than rotavirus infection alone. Such infection resulted in marked clinico-pathological and immunological alterations in the infected piglets.

Keywords

Escherichia coli, India, Pig, Rotavirus, Virus.

Infezione sperimentale in suini con *Rotavirus* del gruppo A ed *Escherichia coli* enterotossico in India: studio delle alterazioni macroscopiche, istopatologiche e immunopatologiche

Riassunto

Gli autori descrivono uno studio condotto ad Assam, India, sulle alterazioni macroscopiche, istopatologiche e immunopatologiche riscontrate in

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suini infettati sperimentalmente con Rotavirus *ed* Escherichia coli *enterotossico (ETEC) con espressione dell'antigene K88. Trenta suinetti, nati con parto cesareo, sono stati infettati sperimentalmente con* Rotavirus *o con* Rotavirus *più ETEC al fine di evidenziare le alterazioni macroscopiche, istopatologiche e il pattern di distribuzione dei differenti sottogruppi di cellule B e T nell'intestino. Negli esemplari infettati con* Rotavirus *è stata osservata atrofia dei villi, soprattutto nel digiuno e nell'ileo, negli esemplari infettati associando* Rotavirus *ed ETEC è stata osservata una grave necrosi dei villi intestinali. Gli studi ultrastrutturali hanno evidenziato simili alterazioni patologiche nell'ileo dei suinetti infetti. L'esame morfologico dei villi e delle cripte intestinali ha rivelato una riduzione nel rapporto tra altezza media dei villi e profondità delle cripte (rapporto VH:CD) sia nel gruppo infettato con* Rotavirus *(5,95 ± 0,33) che in quello infettato con* Rotavirus *ed ETEC (7,90 ± 0,16). Una riduzione più marcata (p<0,01) del rapporto VH:CD è stata osservata nel digiuno (8,83 ± 0,79) e nell'ileo (8,46 ± 0,78) rispetto al duodeno (10,03 ± 0,50). Negli esemplari infettati con* Rotavirus *e sacrificati al sesto giorno* post *infezione è stata riscontrata la presenza di cellule cIgA⁺ nella lamina propria dei villi e di cellule T CD8⁺ negli epiteli dei villi. Nei suinetti l'infezione da* Rotavirus *associata a ETEC è risultata più grave della sola infezione da* Rotavirus*, determinando marcate alterazioni clinico-patologiche e immunologiche.*

Parole chiave

Escherichia coli, India, *Rotavirus*, Suino, Virus.

Introduction

Among the various aetiological agents, rotavirus is one of the major causal agents of mild to severe gastroenteritis and diarrhoea in many animal species including children. In young pigs, rotavirus has been identified as the principal cause of diarrhoea and super-infection. Several studies have revealed rotavirus as the principal and independent cause of piglet diarrhoea (4, 8, 21). However, in a few cases, rotavirus and other entero-pathogens, such as enterotoxigenic *Escherichia coli* (ETEC), have been recognised as combined causative agents of diarrhoea in piglets. Combined infection with *E. coli* resulted in efficient colonisation and development of protracted diarrhoea (23). In our previous study, an incidence rate of 52.3% pathogenic *E. coli*-related diarrhoea was recorded in the organised farms of Assam (3, 29).

Diarrhoea causes significant economic losses to the pig husbandry due to retarded growth, piglet mortality and expenditure involving medical treatment and prophylaxis (30). Animal models, such as mice (22), rabbits (10) and calves (35), have been used extensively to study rotavirus induced gastroenteritis. Given the similarities of the intestinal anatomy and physiology to that of human infants, the colostrum deprived, artificially reared neonatal pig has been considered to be the most appropriate animal model to study the pathobiology of rotavirus infection in humans (13, 17). The pathology of rotavirus infection is restricted to the small intestine (12, 28).

Rotavirus, in association with ETEC, produces more severe pathological alterations in the intestine (33) in comparison to that in mono-infections of piglets with either agent. Alteration at different histotopographic areas, along with ultrastructural studies, can provide a better insight on the pathobiology of the virus in the gut. Immune interaction of rotavirus infection with gut lymphoid cells has not been completely elucidated.

It is reported that B-cells play an important role in clearing primary infection and such lymphoid cells are absolutely necessary for the development of immunity against re-infection with rotavirus (15). The involvement of cell-mediated immunity in rotavirus infection has also been reported (27). However, the association of immunoglobulin isotypes and T-cell subsets in rotavirus infection requires extensive studies that would result in more effective immunoprotective strategies.

The present study highlights the gross, histological and immunopathological alterations in Caesarean derived piglets infected experimentally with rotavirus alone as well as in combination with ETEC.

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Materials and methods

Experimental animals

A total of 30 Caesarean derived and colostrum deprived piglets were used for the trial. Prior to infection, all piglets were kept under constant observation in a sterile isolation unit for a period of 48 h. Piglets were provided sterile cow's milk daily at the rate of 300 ml per kg body weight and drinking water *ad libitum* throughout the experimental period. Piglets was fed three times a day. Stringent hygienic measures were observed and contact with other animals was avoided.

Experimental design

For experimental infection, K88 pili possessing ETEC and group A rotavirus maintained at the Department of Microbiology, College of Veterinary Science of Assam Agricultural University in Khanapara were used. The piglets that had been deprived of colostrum and which were free from rotavirus and ETEC (as screened by enzyme-linked immuno-sorbent assay [ELISA]) were divided into three groups (groups 1, 2 and 3) comprising 10 piglets each. Prior to infection, all piglets were kept under fasting for 4 h-5 h. Piglets in group 1 were inoculated with 2 ml of 20% bacteria-free piglet's intestinal suspension containing 3.2×10^4 $TCID_{50}$/ml orally according to the method described by Debouck and Pensaert (13). To ensure total absorption of the virus, the inoculum was mixed with 10 ml of sterile cow's milk and drenched orally. Piglets from group 2 were infected orally with 2 ml bacteria-free 20% intestinal suspension containing rotavirus and 10 ml of ETEC suspension containing approximately 1.2×10^9 colony-forming units (cfu)/ml (11). The volume of the virus inoculum was increased by mixing it with 10 ml of sterile cow's milk. The piglets of group 3 were kept as uninfected controls and received 2 ml of sterile phosphate buffer saline (PBS) mixed with 10 ml of sterile cow's milk. Five piglets from each group were sacrificed on day 2 post infection (pi) and the five remaining piglets from groups 1 and 3 were sacrificed on day 6 pi. No piglet from group 2 could be maintained up to day 6 pi as all five remaining piglets died on day 2 pi. All sacrificed and dead piglets were immediately subjected to pathological examination. The intestinal content was processed for detection of the inoculated rotavirus and for ETEC.

Detection of rotavirus and *Escherichia coli* in faecal samples

The faecal excretion pattern of rotavirus in faeces was studied using a sandwich ELISA (36) and polyacrylamide gel electrophoresis (PAGE) (19). K88 pili possessing ETEC was identified using an indirect ELISA with specific monoclonal antibodies (MAbs) as described by Barman *et al.* (3).

Histopathological examination

Intestinal tissue from the duodenum, jejunum and ileum were collected from the experimentally infected piglets. For histo-pathological examination, tissues were processed and stained in accordance with the procedure described by Luna (24).The morphometry of the intestinal villi and crypt was studied in the histological sections under a light microscope equipped with a 10× objective and ocular micrometer as described by Moon *et al.* (26) and Crouch and Woode (12). The severity of the lesions in the duodenum, jejunum and ileum was estimated as the ratio between average villus height (VH) and average crypt depth (CD). Five randomly selected well oriented villi and their associated crypts at each position, both in infected as well as in uninfected control animals, were evaluated. Scanning electron microscopy (SEM) of the ileal tissues collected at necropsy from representative animals of groups 1, 2 and 3 were performed to study the alteration due to rotavirus and ETEC infection.

Immunopathological study

The distribution of B and T lymphocytes in different histotopographic areas of the small intestine (duodenum, jejunum and ileum) of all piglets was demonstrated in cryosections using a panel of MAbs against CD2, CD4, CD8, IgA and IgM as described by Lunney and Pescovitz (25) and Van Zaane and Hulst (37). In the non-lymphoid areas, the cells positive to

B and T MAbs were evaluated in the lamina propria as well as in the epithelia. A semi-quantitative estimation of intra-epithelial positive cells was performed per 50 absorptive epithelial cells. The experimental design was approved by the Institutional Animal Ethics Committee (IAEC) of the College of Veterinary Science at the Assam Agricultural University in Khanapara, Guwahati.

Results

Gross changes

In our study, diarrhoea was observed in all piglets that had been infected with rotavirus alone or in combination with ETEC. However, the severity of diarrhoea was greater in the animals that had received rotavirus and ETEC (group 2) where the animals had died on day 2 pi. Piglets from groups 1 and 2 showed major macroscopic changes, primarily in the small intestine. At necropsy, the distal half to two-thirds of the small intestine was dilated with a large volume of yellow to grey watery materials. The intestinal wall appeared thin and flaccid. The stomach of group 1 piglets was distended with undigested milk. Haemorrhagic gastric mucosa was observed in group 2 piglets. Mesenteric lymph nodes (MLN) were slightly congested. The gross changes in the intestine were more intense on day 2 pi than on day 6 pi in group 1 piglets. Animals infected simultaneously with rotavirus and ETEC (group 2) showed severe haemorrhagic gastroenteritis with blood-tinged gut content. MLN also appeared severely haemorrhagic. Piglets from the control group (group 3) exhibited no detectable pathological changes.

Histopathological alterations

Histopathological changes within the jejunum and ileum in the piglets infected with rotavirus were pronounced. However, changes were not marked in the duodenum of the infected piglets. Atrophy of the intestinal villi (Fig. 1) was marked in piglets that had received rotavirus infection alone. In both groups of infected pigs, elongation of the crypts, moderate to severe congestion of the lamina propria and mild infiltration of the intestinal mucosa by polymorphonuclear cells were observed. Numerous desquamated epithelial cells were recorded within the lumen of the jejunum and ileum. In group 1 piglets, histopathological changes were more pronounced in the piglets that were sacrificed on day 2 pi in comparison to those sacrificed on day 6 pi. Piglets infected simultaneously with rotavirus and ETEC (group 2) showed a marked congestion of the mucosa and sub-mucosa. Severe coagulative necrosis and sloughing of the villous ephithelia were recorded. Mild to moderate degrees of crypt hyperplasia were seen (Fig. 2). Villous atrophy was not marked in group 2 piglets. The control animals (group 3) sacrificed on days 2 and 6 pi showed no observable microscopic lesions.

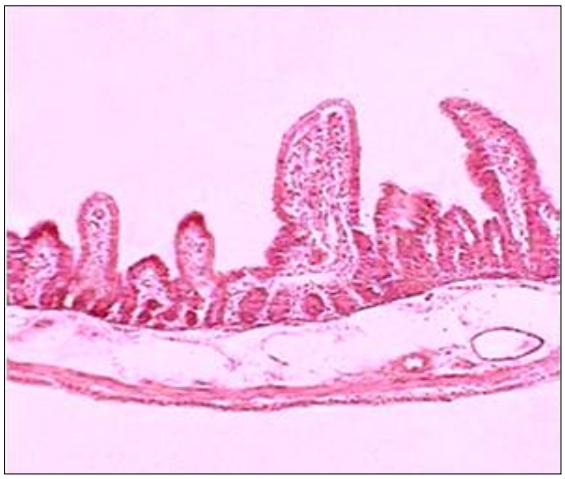

Figure 1
Intestinal section of a piglet infected with rotavirus (group 1) showing marked atrophied villi
(×100)

The morphometry of the intestinal villi and crypts showed alteration of the VH:CD ratio in the duodenum, jejunum and ileum of infected piglets (groups 1 and 2). The mean VH, CD and VH: CD ratio recorded in the duodenum, jejunum and ileum in the different groups of piglets, sacrificed on days 2 and 6 pi are presented in Table I. A statistical analysis revealed that the overall mean VH: CD ratio in piglets from group 1 (5.95 ± 0.33), group 2 (7.90 ± 0.16) and group 3 (12.87 ± 0.31) differed significantly ($p<0.01$). Again, between the

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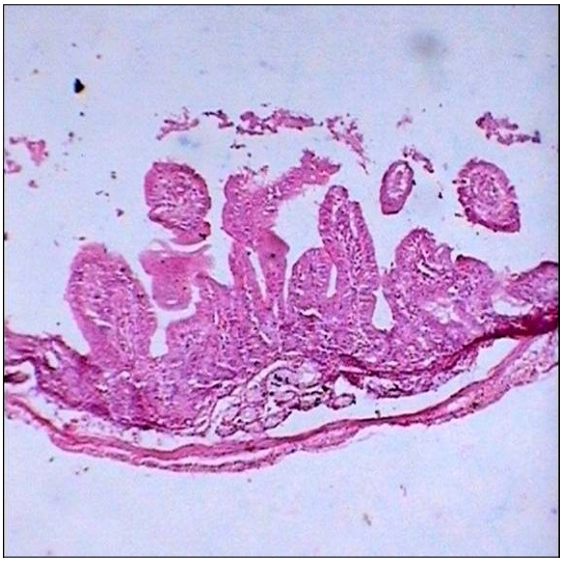

Figure 2
Intestinal section of a piglet infected with rotavirus and enterotoxigenic *Escherichia coli* (group 2) showing severe necrosis and sloughing of villi
(×100)

infected groups (groups 1 and 2), the overall mean VH: CD ratio was significantly (p<0.01) lower in group 1. The morphometric alteration at different parts of the intestine revealed a significantly lower (p<0.01) mean VH:CD ratio in the jejunum (8.83 ± 0.79) and ileum (8.46 ± 0.78) than that of the duodenum (10.03 ± 0.50). However, no such significant difference in the overall means VH: CD ratio was observed in the control piglets (group 3) at different days pi.

Scanning electron microscopy

SEM of the necropsy sections of the ileum in control piglets (group 3) revealed numerous regularly arranged finger-like villi on the intestinal mucosa (Fig. 3, inset). The surface of the villi appeared relatively smooth and was crossed with few transverse furrows. Hexagonal enterocytes were observed on the apical border of the villi. Openings of goblet cells were prominent in most villi and mucous secretions of varying degrees were observed on the villi tips. The ileal tissues of rotavirus infected piglets (group 1) showed marked villous atrophy with blunting of the villus tips (Fig. 3). The villi were rudimentary and appeared bud-like in structure. The length of the villi was reduced to about one-third to one quarter of the normal length of ileal villi. As a result, the associated crypt areas appeared distinct. The normal finger-like structure of the villi was lost and their quantity was found to be less numerous. The enterocytes appeared swollen occluding the transverse furrows and openings of the goblet cells. In most villi, degeneration and detachment of the enterocytes from lamina propria was observed.

Table I
Mean ± standard error of villus height, crypt depth and villus height: crypt depth ratio in different parts of small intestine among piglets at different days post inoculation

Group	Type of inoculum	Day of sacrifice	Duodenum			Jejunum			Ileum		
			VH	CD	Ratio	VH	CD	Ratio	VH	CD	Ratio
1	Rotavirus	2	466.50 ± 15.02	58.41 ± 2.10	7.99 ± 0.12	365.74 ± 8.65	87.52 ± 3.75	4.19 ± 0.09	380.82 ± 6.67	112.83 ± 4.87	3.39 ± 0.09
		6	546.84 ± 4.65	66.73 ± 0.84	8.19 ± 0.10	428.58 ± 14.98	69.70 ± 2.44	6.14 ± 0.06	433.34 ± 9.61	75.08 ± 2.53	5.78 ± 0.06
2	Rotavirus + ETEC	2	479.02 ± 11.07	56.63 ± 2.31	8.48 ± 0.21	454.97 ± 7.94	58.32 ± 0.97	7.79 ± 0.03	462.38 ± 13.02	68.96 ± 4.38	7.41 ± 0.28
		6	–	–	–	–	–	–	–	–	–
3	Sterile PBS	2	516.76 ± 6.06	41.38 ± 2.56	12.68 ± 0.78	551.65 ± 8.33	43.59 ± 3.12	12.90 ± 0.93	548.18 ± 10.90	42.85 ± 1.73	12.83 ± 0.33
		6	569.07 ± 5.04	45.24 ± 2.86	12.79 ± 0.86	592.77 ± 5.10	46.67 ± 4.09	13.12 ± 1.25	588.35 ± 5.13	45.89 ± 1.32	12.86 ± 0.38

VH villus height
CD crypt depth
ETEC enterotoxigenic *Escherichia coli*
PBS phosphate buffered saline

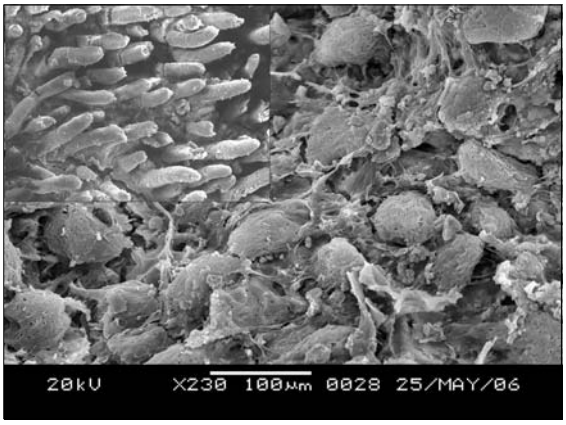

Figure 3
Intestinal villi of a piglet infected with rotavirus showing marked villous atrophy with blunting of the villus tips under scanning electron microscopy
Inset: Scanning electron micrograph of intestinal villi of healthy uninfected control piglet

As a result, there were numerous holes on the villus surfaces. The affected villi surface had lost the usual smooth and even contour. Piglets infected simultaneously with rotavirus and ETEC (group 2) showed severe degenerative and necrotic changes of the ileal villi (Fig. 4). The villi had lost their normal appearance and only a few intact villi were observed. Most intact villi showed mild to moderate degrees of atrophy. Severe necrosis and desquamation of the villus enterocytes were observed on the upper one-third of the villi. The affected villi surface appeared rough and the transversal furrows and openings of the goblet cells were indistinct. Villus surfaces were covered with numerous rod-shaped bacteria (Fig. 5).

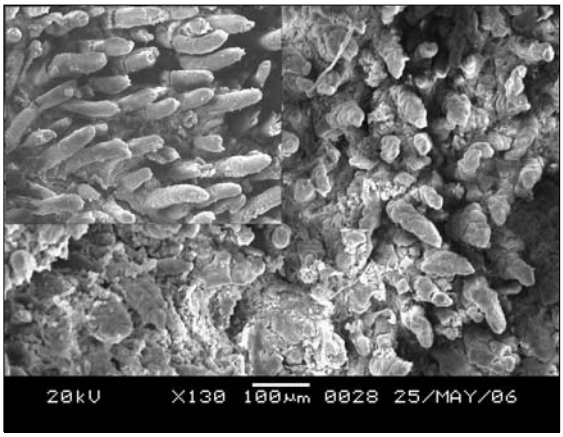

Figure 4
Scanning electron micrograog of illial villi of a piglet infected simultaneously with rotavirus and enterotoxigenic *Escherichia coli* showing severe degenerative and necrotic changes
Inset: Intestinal villi of healthy uninfected control piglet under scanning electron microscopy

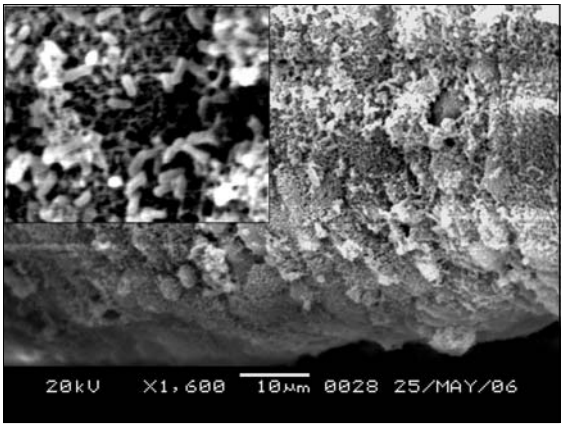

Figure 5
Intestinal villi of a piglet infected with rotavirus and enterotoxigenic *Escherichia coli* (ETEC) showing showing aggregation of numerous rod-shaped bacteria under scanning electron microscopy
Inset : Magnified rod-shaped bacteria (ETEC) attaching onto villi

Immunohistology

The distribution of B- and T-cell subsets in lymphoid and non-lymphoid areas of all experimental animals was demonstrated in cryosections of duodenum, jejunum and ileum. The lymphoid areas in duodenum, jejunum and ileum of the infected (groups 1 and 2) and the control (group 3) piglets stained with IgA MAbs showed different staining reactions. Entire follicles and the dome area in the infected piglets showed network-like staining reactions (Fig. 6), whereas in the control animals, only surface positive cells were observed. Again, staining of the lymphoid areas in the infected and the control animals with IgM-specific MAb showed a surface positive staining reaction in the follicles and in the dome of all the three regions of the intestine.

Cryostat sections, stained with T-cell-specific MAbs showed the distribution of T-cell subsets in the inter-follicular area (IFA) as well as in the dome of the follicles in the infected (groups 1 and 2) and control (group 3) piglets.

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CD2+ T-cells were evenly distributed in the entire IFA and in the dome areas. Variation in the positively stained cellular intensity was not marked between infected and the uninfected piglets. In the infected piglets, about two-thirds of the IFA revealed the presence of CD4+ cells. However, in the dome area, the distribution of CD4+ cells was also scattered. The distribution pattern of CD4+ cells was comparable in infected and non-infected piglets. The distribution of CD8+ T-cells was limited to one-third of the IFA in the case of infected animals (Fig. 7). They were also found in the dome areas in the infected piglets. In the control animals, there was scanty distribution of CD8+ cells in the IFA.

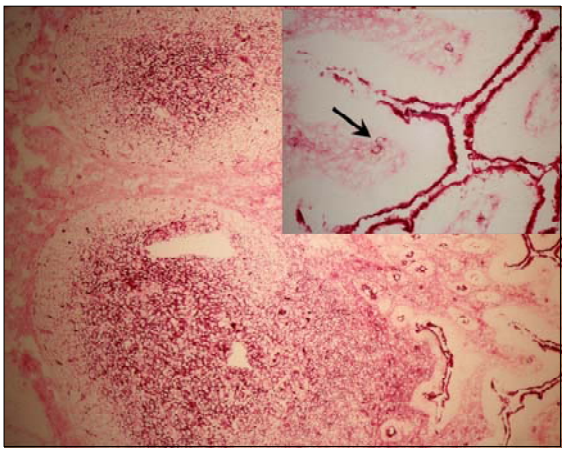

Figure 6
Cryosection stained with IgA monoclonal antibody showing a network-like staining reaction in the follicle
(×1 000)
Inset: Lymphocytes containing cytoplasmic IgA (cIgA) in the villus lamina propria (×400)

Distribution of different B- and T-cell subsets in the non-lymphoid areas of the intestine showed variations among infected and control piglets. Among the B-cell subsets, surface positive IgM (sIgM+) cells were distributed in the lamina propria of the villus and in the crypt of both infected and control groups. Cytoplasm containing IgA+ (cIgA+) cells appeared in the lamina propria of villi (Fig. 6, inset) and crypts of infected animals, particularly in the animals with rotavirus that were sacrificed on day 6 pi.

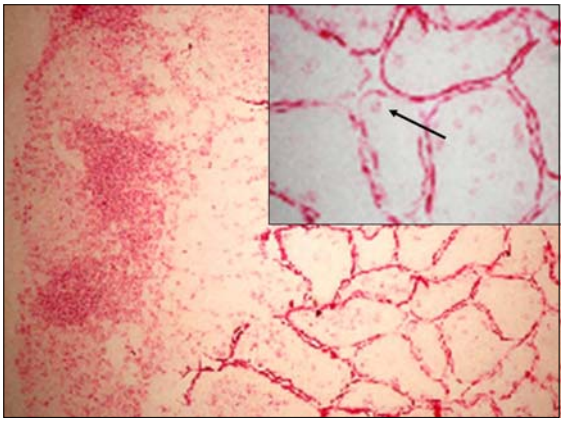

Figure 7
Cryosection stained with CD8 monoclonal antibodies showing cytotoxic T-cells in the inter-follicular area and villus lamina propria
(×1 000)
Inset: Intra-epithelial CD8+ cells in villus epithelia (×400)

Evaluation of the T-cell distribution in the non-lymphoid areas of the intestine revealed the presence of T-cells in the lamina propria of villi and crypts in both infected and control groups. Most of the lymphocytes in the lamina propria were stained with CD2+ MAbs. In the infected piglets, CD2+ cells were occasionally observed in the intra-epithelial area of villi. However, in uninfected healthy piglets, no CD2+ cells were observed in the intra-epithelial area. Presence of CD4+ cells was demonstrated only in the lamina propria and no marked difference in the distribution of the cells was observed between infected and non-infected piglets. CD8+ cells were observed in the lamina propria of villi and crypts in both infected and non-infected piglets. Cryosections of the intestine in piglets infected with rotavirus (group 1) sacrificed on day 6 pi revealed the presence of intra-epithelial CD8+ cells (Fig. 7, inset) in the villus areas. A semi-quantitative estimation of CD8+ intra-epithelial T-cells revealed 4 to 5 positive cells per 50 absorptive epithelia. No CD8+ intra-epithelial T-cells were observed in the uninfected animals (group 3) and in the piglets infected with both rotavirus and ETEC (group 2).

Discussion

Rotavirus is an enterotropic virus and the infection is mostly restricted to the small intestine (31). In our investigation, we recorded a severe form of gastroenteritis and haemorrhagic lesions in the group of animals infected with rotavirus (group 1) as well as in the group infected with both rotavirus ETEC (group 2). The same results were reported by Theil *et al.* (32) and Collins *et al.* (9) in piglets infected with rotavirus. Piglets infected simultaneously with rotavirus and ETEC showed macroscopic changes in the stomach, intestine and MLN. However, the haemorrhagic and inflammatory changes in this group of piglets were more pronounced compared to the piglets infected with rotavirus alone. Such changes were attributable to the enterotoxic effect of ETEC (14, 16).

Rotavirus replicates predominantly in the cytoplasm of differentiated small intestinal villous epithelial cells and thus the virus induces histopathological changes that are restricted to the small intestine (12, 29). The microscopic changes include the degenerative consequences of rotavirus-induced villous epithelial cell destruction and the adaptive and regenerative responses of the small intestine. In our study, the piglets infected with rotavirus (group 1) showed marked villous atrophy and crypt hyperplasia. Besides, coagulative necrosis of the villi and moderate to severe congestion of the lamina propria, muscularis mucosa of the small intestine were observed. Theil *et al.* (32) and Gomez *et al.* (18) also reported the occurrence of severe villous atrophy and crypt hyperplasia in the intestine of piglets infected with rotavirus. The intestinal villus atrophy observed in the present study might have resulted from rotavirus-induced villus epithelial cell degeneration and desquamation (28). Hyperplasia of the cell lining of the crypts might have occurred in an effort to retain the normal villous structure in the face of massive epithelial cell destruction (12). SEM of the ileal tissues in the piglets infected with rotavirus revealed marked villus atrophy associated with severe degenerative changes of the enterocytes. The affected villi were rudimentary and appeared bud-like in structure. These changes further depicted the virus-induced pathological alterations in the intestine of the piglets infected with rotavirus. Torres-Medina and Underdahl (33) also reported marked villus atrophy with blunting of the villus tips in the ileum of piglets infected with rotavirus. Evaluation of histopathological alterations in different parts of the intestine (duodenum, jejunum and ileum) showed prominent microscopic lesions in the jejunum and ileum of the piglets infected with rotavirus. This observation reflects the tropism of the virus towards these regions. However, limited involvement of the duodenum might have been the result of differences in the cell differentiation in this part of the small intestine and possibly due to the greater abundance of inhibitory agents in the upper part of the gut (12). A histopathological study of the intestine in group 1 piglets showed a mild degree of polymorphonuclear cell infiltration in the intestinal mucosa. These inflammatory changes clearly indicated the occurrence of an acute viral infection in the piglets.

The histopathological changes observed on day 2 pi in piglets simultaneously infected with rotavirus and ETEC (group 2) were found to be much more severe, in comparison to those of the piglets infected with rotavirus alone (group 1). Severe necrosis and desquamation of the villus enterocytes were recorded in this group of piglets, along with marked congestion of the intestinal mucosa and sub-mucosa. Similar findings were reported by Tzipori *et al.* (34). The severe necrotic and degenerative lesions of the villus enterocytes in group 2 piglets suggested the predominance of the enterotoxin. Propagated rotavirus in the gut might alter the integrity of the enterocytes and could have facilitated greater colonisation of ETEC in the intestinal villi (23). A SEM study demonstrated coating of the entire villus surface with rod-shaped bacteria. However, enterotoxins released by ETEC that adhered to the villous surface resulted in the development of vascular congestion, haemorrhages and infiltration of leucocytes in the lamina propria (14). The

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effect of rotavirus on villi in this group was less significant. Benfield *et al.* (5) reported that villus atrophy was less severe in 3-day-old piglets infected with rotavirus and ETEC than in pigs inoculated with rotavirus alone. The results of our morphometric study clearly showed that the VH:CD ratio was not significantly reduced in animals infected with both rotavirus and ETEC. On the other hand, there was a significant reduction of the VH:CD ratio in piglets infected with rotavirus alone. Thus ETEC played a major role in damaging the intestinal integrity through enterotoxins and probably caused the death of piglets within 48 h pi.

Prior characterisation using a mice pathogenicity test and rabbit ligated ileal loop study confirmed the pathogenic and enterotoxigenic nature of the ETEC isolate. SEM of the ileum in group 2 piglets also revealed severe degenerative and necrotic lesions. These ultrastructural changes correlated well with the histopathological lesions and indicated the predominance of ETEC infection in group 2 piglets. The histopathological changes observed in the jejunum and ileum of the simultaneously infected piglets were more pronounced in comparison to those in the duodenum (14, 16).

A morphometric analysis of the villi in duodenum, jejunum and ileum and their associated crypts in the piglets infected with rotavirus (group 1) and those infected with rotavirus and ETEC (group 2) showed a reduction in the VH and increase in the CD compared to the control piglets (Table I). These alterations in VH and CD resulted in a significant reduction in the VH:CD ratio in the infected groups (groups 1 and 2). Furthermore, among the infected groups, the VH:CD ratio in piglets infected with rotavirus (5.95 ± 0.33) was found to be significantly lower than that in the piglets infected with rotavirus and ETEC (7.90 ± 0.16). Crouch and Woode (12) reported similar findings in piglets infected with rotavirus and recorded a VH:CD ratio as low as 4:1 in the middle and distal part of the intestine.

In the present study, the reductions in the VH in the infected piglets might have resulted from the severe degenerative changes of the villus enterocytes (28, 33). The increased crypt depth, on the other hand, might have been caused by an effort to maintain the normal villus structure (12). An analysis of the alterations of VH:CD ratio in different parts of the intestine revealed that that the ratio was significantly lower in the jejunum (8.83 ± 0.79) and ileum (8.46 ± 0.78) than that in the duodenum (10.03 ± 0.50) of the infected piglets. These differences might be due to the variation in cell differentiation or possibly the variability in concentration in inhibitory agents in the different parts of the small intestine (12).

It is interesting to note that the piglets infected with rotavirus sacrificed on day 2 pi showed more severe histopathological changes than that those sacrificed on day 6 pi. Several rotavirus inoculation studies in neonatal pigs have shown that the incubation period of the disease is short and the histopathological lesions, predominantly villus atrophy and crypt hyperplasia, are most severe 24 h-72 h of infection (28, 31). The present findings clearly corroborate the observations made by Theil *et al.* (32) and Rhoads *et al.* (28). In a morphometric analysis, piglets infected with rotavirus and sacrificed on day 2 pi (5.19 ± 0.54) showed a significantly lower VH:CD ratio than those sacrificed on day 6 pi (6.71 ± 0.29). This clearly indicates the occurrence of pronounced pathological alterations in the intestine of piglets sacrificed on day 2 pi. The higher VH:CD ratio in piglets sacrificed on day 6 pi might be due to the loss of viral receptor sites (20) following replacement of the damaged villus epithelium by undifferentiated cells (12).

Development of various immunological compartments in the intestine of pigs occurs in the perinatal period. Various studies have shown the immunocompetency of the B- and T-cell subsets localised in different histotopographic areas of the pig intestine (2, 7). In the present study, the cryosections of duodenum, jejunum and ileum of uninfected piglets (group 3) showed the presence of surface positive IgM ($sIgM^+$) lymphocytes in the follicles and in the domes. The distribution of $sIgM^+$ cells was also scattered in the lamina

propria of villi and crypts. Furthermore, isolated sIgA$^+$ cells were observed in the follicles and in the domes of all three regions of the intestine. Similar observations were made by Barman *et al.* (2) in germ-free pigs aged one month, where preferentially sIgM$^+$ but fewer IgA$^+$ B–cells were observed in the follicles, domes and dome epithelia. Again, a study of the distribution pattern of T-cell subsets in the duodenum, jejunum and ileum of the control pigs (group 3) showed intensely stained CD2$^+$ T-cells in the IFA, dome areas and also in the lamina propria of villi and crypts. However, CD4$^+$ and CD8$^+$ cells were sparsely distributed in these compartments. Furthermore, the distribution of CD8$^+$ cells in these areas was found to be scanty compared to that of the CD4$^+$ cells. A similar distribution pattern of the different B- and T-cell subsets was recorded by Bianchi *et al*. (6) in unprimed animals.

In the present study, animals infected with rotavirus (group 1) and rotavirus and ETEC (group 2) and sacrificed on day 2 pi revealed no marked alteration in the distribution of gut-associated B- and T-cell subsets. However, piglets infected with rotavirus (group 1) sacrificed on day 6 pi showed a marked alteration in the distribution of gut-associated B- and T-lymphocytes. Staining of the intestinal cryosections with IgA MAbs in these piglets showed an intense network-like staining reaction in the follicles. This clearly indicated the activation of the follicles in response to viral infection (2). Again, the appearance of cytoplasm containing IgA$^+$ (cIgA$^+$) cells in the lamina propria of villi and crypts in these piglets indicated switching of IgM$^+$ cells to cIgA$^+$ cells in response to the acute rotavirus infection. The present study therefore suggests that the secretory IgA immune response could be generated as early as day 6 pi to clear rotavirus infection in natural cases. However, B-cell response was not marked in piglets infected by rotavirus that were sacrificed on day 2 pi as well as in piglets that died after simultaneous infection with rotavirus and ETEC. Such a short duration of active infection was probably not optimum for the switching of immune associated cells.

A study of the distribution pattern of T-cell subsets in the intestine of piglets infected with rotavirus as well as in piglets infected with rotavirus and ETEC 48 h pi was comparable with the healthy control piglets. No T-cell positive lymphocytes developed in the intra-epithelial area. However, piglets sacrificed on day 6 pi revealed the presence of T-cell positive intra-epithelial lymphocytes (CD8$^+$ cells) in the villus epithelia. A semi-quantitative study showed exclusive recruitment of CD8$^+$ T-cells into the intra-epithelial zone of the enterocytes in the group infected with rotavirus, compared to uninfected controls as well as to piglets infected with both rotavirus and ETEC. Again, the absence of intra-epithelial CD8$^+$ phenotypes 48 h pi with rotavirus suggests a definite time period for recruitment of lymphocytes after interacting virus with the enterocytes. Various studies indicate the involvement of cytokine-mediated development of intra-epithelial lymphocytes. It has been reported that intra-epithelial lymphocytes developed in the presence of gut antigens and were predominantly of CD8$^+$ phenotype in pigs (1). These findings reaffirm that the development of intra-epithelial lymphocytes is antigen dependent.

The present study suggests that the simultaneous infection of piglets with rotavirus and ETEC results more severe disease than in cases of infection with rotavirus alone. Both humoral and cell-mediated immune response play a significant role in protecting gut mucosa from rotavirus and ETEC infection.

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