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LIGHT AND ELECTRONIC MICROSCOPIC STUDIES
OF THE CHANGES IN THE INTESTINAL MUCOSA
OF GNOTOBIOTIC CALVES INFECTED
WITH A WILD ROTAVIRUS

by H. C. Dubourguier, O. Mandard, M. Contrepois and P. Gouet

Laboratoire de Microbiologie, INRA,
CRZV de Theix, 63110 Beaumont (France)

SUMMARY

Experimental infection of four gnotobiotic calves by a rotavirus isolated from the faeces of diarrheic calves caused non-fatal diarrhoea without dehydration. Lesions of the alimentary mucosa appear well before the onset of diarrhoea and are characterized by a release of mucus in the anterior part of the intestine. By the time diarrhoea begin, different types of rotavirus particles are present in the enterocytes of the mucosa, and mucus contents of distal intestine are affected. At this stage of the disease, the virus causes epithelial cells in the small intestine to desquamate and changes the secretion of mucus in the small as well as the large intestine. The very intense virus multiplication is completed within 18 h. Intracellular rotavirus disappear very quickly after the onset of diarrhoea, and cannot be detected even during the acute phase of the disease. Regeneration of the mucosa takes place slowly, and the cellular and structural lesions are still visible more than 4 days after the diarrhoea stopped.

Key-words: Rotavirus, Diarrhoea, Calf, Intestine; Mucus.

INTRODUCTION

The presence of rotavirus has been shown in many cases of neonatal diarrhoea, in man [1], calves [8, 14], piglets [18] or lambs [16]. A strain of bovine rotavirus, isolated in the USA by Mebus et al. [8] was shown to be pathogenic in gnotobiotic calves deprived of colostrum [9]. The wave of infection affects the entire small intestine and may proceed
from the duodenum to the ileum [9, 15]. In similar experiments, using a strain of rotavirus isolated in France, Gouet et al., have shown that diarrhoea is transient, does not result in dehydration and that severity varies according to the age of the animals [6].

This report describes the development of the lesions of the intestinal mucosa at the time of experimental infection of the gnotobiotic calves with rotavirus, and particularly alterations in quantities of mucus.

**MATERIALS AND METHODS**

All the calves used in these experiments were delivered and reared according to gnotobiological techniques. Seven gnotobiotic calves were delivered either by aseptic caesarian section [13], or by septic caesarian section and transfer to an isolator through an antiseptic liquid chamber [4], or *per vaginam* with decontamination by antibiotics [5] (table I). Three of the calves were not inoculated and served as control. The wild rotavirus was supplied by Dr Scherrer and inoculated as previously described [6]. Using general anaesthesia, samples for histological examination were obtained at different times after inoculation from the following location of the gastro-intestinal tract: the antral area of the abomasum, duodenum (distal to the pancreatic duct), middle jejunum, ileum (50 cm before the ileo-caecal valve), caecum (opposite the ileal-caecal valve) and spiral colon.

**Table I. — Source, method of inoculation and occurrence of diarrhoea in gnotobiotic calves.**

| Calf | Source of calves | Age at inoculation | Age at sampling | Age at appearance of diarrhoea | Contamination observed at the time of autopsy |
|------|------------------|--------------------|----------------|-----------------------------|---------------------------------------------|
| 1    | *Per vaginam*    | Control            | 12             | --                          | Germ-free                                  |
| 2    | *Per vaginam*    | Control            | 21             | --                          | Fine bacilli                               |
| 3    | Septic caesarian | Control            | 120            | --                          | 6 × 10⁶ *Clostridium* /g of faeces          |
| 4    | Septic caesarian | 2                  | 12             | --                          | Germ-free                                  |
| 5    | *Per vaginam*    | 1.5                | 21             | 20                          | Germ-free                                  |
| 6    | *Per vaginam*    | 2                  | 28             | 17                          | Germ-free                                  |
| 7    | Aseptic caesarian| 27                 | 8 days (*)     | 46                          | Fine bacilli, streptococci                 |

(*) 111 h after the end of diarrhoea.

Samples were immediately and carefully rinsed in Sörensen's phosphate buffer 0.1 M, pH 7.2 prior to fixation. For transmission electron microscopy (TEM) samples were fixed either by double fixation method (calves 1, 2, 3, 5 and 7) or by 1 % osmium tetroxide in a phosphate buffer pH 7.2 for 1.5 h. After dehydration in ethanol and propylene oxide the tissue fragments were embedded in Epoxy

LM = light microscopy.  
PAS = periodic acid Schiff.  
SEM = scanning electron microscopy.  
TEM = transmission electron microscopy.
resin (Epikote 812, Touzart et Matignon, 94400 Vitry-sur-Seine, France). Sections
were cut to an average thickness of 600 Å and were stained with uranyl acetate
alcohol and citrate. Observations were carried out using a « Jeol » electron micros-
cope (Jeol 100B) with an acceleration potential of 80 kV. Analysis of intestinal
contents was performed according to the technique used by Snodgrass et al. [16].
For scanning electron microscopy (SEM), samples were prepared as previously
reported [3]. For light microscopy (LM), samples were fixed in Baker's formol
mixture, cut into 7 µm sections and stained either with Giemsa or Alcian blue-
PAS with a general eosin staining.

Histological analysis of the quantity of mucus present in the tissues was
carried out on the sections stained with Alcian blue-PAS. This analysis was per-
formed with a « Wild » microscope connected to a surface analyser (prototype
apparatus) which converts the optical image to an electronic signal by a television
camera (I.T.C.). A synthetiser linked to a television screen allows the comparison
of the reconstructed image with the real one, after regulation of the selection
thresholds of analyser. When both images are superposed, the analyser converts
the surface of each selected shade into arbitrary proportional values. It is then
possible to determine the ratio (R) between the surface area of the secretory
granules of the goblet cells (stained purple by the Alcian blue-PAS) and the total
surface area of the intestinal tissue (stained pink by the eosin). In this manner,
measurements are not made by image intensity, but only by surface area. Surfaces
areas of the goblet cells and therefore their volume, are related to their mucus
content.

Bacteriological tests were performed on the faeces of the animals. Samples
were examined microscopically, stained by the Gram stain and inoculated on
the following media: tryptiease soja broth (B. D. Merieux) and agar slant, thiogly-
collate broth (Difco) inoculated before and after heating the faeces at 75°C for
10 min. The incubation was made at 37°C and results checked after 24, 48, 72 h
and after 1 week.

RESULTS

Of the 7 calves used, 4 of them remained germ-free, and non-pathogenic
bacteria were found in the other 3 (table I).

The control calves remained normal and were sacrificed when 12, 21
and 120 h old (table I). With microscopic technics, the 3 control calves
were similar as early reported [3].

In the duodenum, goblet cells were seen between the enterocytes. Mitochondria and Golgi bodies were common in these cells and much
of the cytoplasm was occupied by secretory granules. In the jejunum and ileum, goblet cells had a similar appearance (fig. 1). The surface area
of goblet cells was smaller in the jejunum (fig. 2). In the cæcum and the
colon, goblet cells were plentiful and their area was two to three times
observed in the jejunum (fig. 3). Results of histo-quantitative analysis
of goblet cells were similar in the 3 control calves except in the cæcum
of the 12 h old calf due to meconium (table II).

In calf nº 4, 10 h after inoculation and before the appearance of diarrhoea, the intestine did not contain any liquid. By light or SEM micros-
copy, the structure of the entire small intestine was normal except for
vacuoles in enterocytes of the ileum.

No rotavirus was observed by TEM in the intestinal contents or in
epithelial cells during this early stage of infection.
TABLE II. — Comparison of the mean values of $R$ at different levels of the intestinal tract of the control (C) and inoculated (I) calves (n°).

|                | Before diarrhoea | Acute phase of diarrhoea | Recovery phase |
|----------------|------------------|--------------------------|---------------|
|                | C1  | I4  | C2  | I5  | I6  | C3  | I7  |
| Duodenum       | 0.112 | 0.092 (*) | 0.109 | 0.098 | 0.206 (**) | 0.107 | 0.127 (**) |
| Jejunum        | 0.083 | 0.062 (*) | 0.078 | Desquama- | 0.107 | 0.086 | 0.050 (***) |
| Heum           | 0.101 | 0.112 NS | 0.108 | 0.064 (***) | 0.053 (***) | 0.112 | 0.086 (*) |
| Caecum         | 0.201 | 0.145 (***) | 0.114 | 0.050 (***) | 0.120 (**) | 0.155 | 0.143 NS |
| Colon          | 0.254 | 0.210 NS | 0.228 | No sample | 0.148 (***) | 0.259 | No sample |

Statistical analysis performed with Student’s test

$R = \frac{\text{surface area of the secretory granules of goblet cells}}{\text{total surface area of the examined section of intestinal tissue}}.$

NS = $P > 0.05$; (*) = $0.01 < P \leq 0.05$; (**) = $0.05 < P \leq 0.01$; (***) = $P < 0.005$.

FIG. 1. — Nucleus of a goblet cell which has released its contents and surrounded by enterocytes.

Jejunum of the control calf n° 3. TEM, $\times 20,000$.

FIG. 2. — Ileal villus in the control calf n° 3, with numerous goblet cells.

Alcian blue-PAS; LM, $\times 250$.

FIG. 3. — Mucosa of the colon of the control calf n° 3.

Observe the high density of the goblet cells along the folds and the almost total disappearance of these cells in the luminal surface. Alcian blue-PAS; LM, $\times 250$.

FIG. 4. — Rotavirus particles in the proximity of the nucleus of a duodenal enterocyte in calf n° 5 in the initial phase of diarrhoea.

The viral particles (arrow) surround a vacuole which appears to have burst. TEM, $\times 20,000$.

FIG. 5. — Jejunal villus in calf n° 5 at the onset of diarrhoea.

Note the protrusions from the apex of each villus on which the enterocytes are in progress of expulsion. SEM, $\times 150$.

FIG. 6. — Ileal enterocyte in calf n° 5 at the onset of diarrhoea.

Note the low cytoplasmic density. Notice the rotavirus inside large basal vacuoles (arrow). TEM, $\times 4,000$.

FIG. 7. — Rotavirus particles inside a basal vacuole which is surrounded by ribosomes.

Calf n° 5. TEM, $\times 80,000$. 
Calf no. 5 became diarrheic 18.5 h post-inoculation and was sampled 1 h later at which time the small intestine was slightly flushed. Rotavirus particles were found in the intestinal contents at each level except that of the abomasum. The abomasum did not show any lesion for an increased number of mast cells in the lamina propria and turgidity of the epithelial cells. In the duodenum, the amount of cellular vacuolisation decreased from the tip to the base of the villi. The nucleus was surrounded by free spherical granules with an homogenous diameter of about 54 nm (fig. 4). The basolateral cytoplasmic membrane was partially destroyed. In the jejunum, the tips of some villi were desquamated (fig. 5) and the enterocytes near the apex of the villi were cuboidal. Cytoplasmic changes of the enterocytes were similar to those observed in the duodenum. No free granules of 54 nm were observed. However a few granules were seen inside vacuoles and were associated with viral particles which had a dark nucleus surrounded by a clear ring, all of which had a total diameter of about 69 nm. Some viral particles of this type were also free in the cytoplasm. In the ileum, the shape of the villi was altered by the release of mucus which reduced the number of goblet cells. In the upper half of the villi, the enterocytes had an irregular brush border and had lost their cytoplasmic contents as well as their nuclei. The cytoplasm, which was very clear, contained mainly vacuoles and polysomes. Vacuoles in the basal region of the cell contained rotavirus (fig. 6) whose diameter varied between 60 and 73 nm (fig. 7). There were no free-virus particles in the cells. The lamina propria contained a few mast cells. Changes were not marked in the cæcum and colon. Intracellular viral particles were not observed. In addition, we never found any virus in goblet cells in small intestine and large intestine.

Calf no. 6 was sampled 26 h after inoculation and 11 h after the onset of diarrhoea at which time the abomasal mucosa was covered by a layer of mucus. The folds of the mucosa were very long, resembling small villi and desquamated at the apex. In the duodenum, the villi were short and very rounded. Numerous eosinophilic polymorphonuclear cells and a few mast cells were present in the lamina propria. The jejunum and the ileum showed similar lesions. The jejunal villi were distorted and swollen by a pronounced infiltration of endothelial cells into the lamina propria. Some villus tips were desquamated and the distal two thirds of most villi were covered with cuboidal cells. The cæcal and colonic mucosa appeared to be normal. No rotavirus particles were seen in epithelial cells at any of the sampling locations; however virus was observed in the cæcal contents.

Calf no. 7 was diarrheic from 19 to 53 h post-inoculation. It was sampled when 8 days old, 111 h after diarrhoea had stopped at which time it was clinically normal. Some abomasal cells at the tips of the folds had expelled their contents. The duodenal villi were distorted, flattened, short and appeared to be regenerating. The outer third of some jejunal villi was desquamated or covered with cuboidal cells and seemed to be soft. The ideal villi were leaf-shaped very indented and their tips covered with
cuboidal cells. Eosinophilic polymorphonuclear cells were visible in the lamina propria. The mucosa of the cæcum and colon was similar to that in the control calf.

Histo-quantitative analysis of the goblet cells (table II) showed that mucus was affected at all levels of the small and large intestines. Before the onset of diarrhoea (calf n° 4), the duodenum and jejunum were affected. The changes reached the ileum and the cæcum by the time diarrhoea begin (calf n° 5) and persisted throughout the acute phase of diarrhoea (calf n° 5 and n° 6) when the large intestine was also affected. Alterations were also observed during the recovery phase as long as 111 h after diarrhoea had ceased (calf n° 7).

DISCUSSION

All samples were taken under general anaesthesia without exsanguination in order to avoid the epithelial desquamation observed during preliminary test (unpublished results) [12]. The double fixation method used for SEM allowed us to observe the surface of samples with as much precision as with the chemical preparation used by Mebus [7] and the physical preparation (critical point) performed by Newman and Trapp [11]. In control calves, there is no change in epithelial cells depending upon age (12, 21 and 120 h old). Only in the cæcum, more mucus was observed in the youngest calf. This might be due to residual meconium.

The rotavirus infection of gnotoxenic calves is not fatal and causes transient diarrhoea without dehydration [6]. The time of onset (15-19 h) and the duration (24 h) of rotavirus diarrhoea agree with our earlier observation [6] and with those of Mebus [7]. During the incubation and initial phases of diarrhoea, a prominent vacuolization of the enterocytes specially in the mucosa of the small intestine was seen. Later in the course of diarrhoea, cells were desquamated and replaced by cuboidal cells. Similar changes have been shown in calves by Mebus et al. [9] and in the piglet suffering from transmissible gastroenteritis [17]. This important vacuolization might correspond to a early cell degeneration, followed by desquamation. Lesions were seen also in the abomasum before the occurrence of diarrhoea and persisted for several days after the disappearance of the clinical symptoms. However, these lesions were probably not a direct result of viral infection since no virus was observed either in the epithelial cells or in the abomasal contents.

Ten hours post-inoculation, no rotavirus was seen in the intestinal cells. Later in the course of diarrhoea, numerous circular granules of 54 nm in diameter were observed. According to Chasey [2] they may correspond to non-infectious incomplete virus particles called type IV particles by Chasey. To our knowledge, type IV particles have never been reported in vivo in the calf. For Chasey this viral type is found in damaged cells and more often in lesser number in small vacuoles [2]. In our experiments, this type of particles were found in great number inside the badly damaged
cells and around the vacuoles which seemed to have burst. At this same stage of infection, we saw complete viral particles with a diameter of 60 to 73 nm both in the epithelial cells of the small intestine and in the intestinal contents. The dimensions are similar to those found by Stair et al. [15]. According to Chasey, this type of particles could correspond to the final stage of maturation of the rotavirus. These different types of particles are before the appearance of diarrhoea. In the acute phase of diarrhoea, no viral particles were observed in the cells, although they were still present in the cecal contents. These results indicate that after infection, the rotavirus multiplies very quickly inside the enterocyte, and its complete maturation must take place in vivo within about 18 h post-inoculation. From the micrographs obtained, the number of viral particles released by infected cells can be estimated at about 10,000.

The gastro-intestinal lesions had not completely disappeared 111 h after diarrhoea had ceased since the tips of some abomasal folds and some intestinal villi were still covered by abnormal cells. This observation suggests that following rotavirus infection epithelial cell replacement time is longer than the 48-72 h observed in normal calves [10]. The renewal time of the lamina propria may increase the apparent replacement time of epithelium. So, in the rotaviral diarrhoea, the digestive and absorptive functions of the cells are altered, which gives rise to the diarrhoea. From the first emission of diarrhoea, the virus is present in each part of small intestine, but not in the large intestine which confirms the observations made by Mebus [7]. The changes in mucus contents are accentuated and are developed concurrently with the wave of infection. But rotavirus particles were never found in goblet cells. So, this alteration in mucus content may probably be related to inflammatory response of the calf. The important expulsion of infected cells, undoubtedly quicker than the migration of cuboidal immature replacement cells, may explain the onset of desquamation in the apexes of the villi. The normal functions of the intestine are definitely not restored before the regeneration of the intestinal epithelium and lamina propria is complete which requires several days. During this period, the change in the digestive functions might increase the sensitivity of the animals to enteropathogenic Escherichia coli, as the results of Gouet et al. [6] might suggest.

RÉSUMÉ

ÉTUDES AUX MICROSCOPES OPTIQUE ET ÉLECTRONIQUE DES MODIFICATIONS DE LA MUQUEUSE INTESTINALE DE VEAX GNOTOBIOTIQUES INFECTÉS AVEC UNE SOUCHE SAUVAGE DE ROTAVIRUS

L’infection expérimentale de quatre veaux gnotoxéniques par un rotavirus sauvage induit une diarrhée non mortelle sans déshydratation.
Les lésions gastro-intestinales apparaissent même avant le début de la diarrhée et sont caractérisées par une diminution de la quantité de mucus présente dans l’intestin grêle. Après l’infection, différents types de particules sont présentes dans les entérocytes de la muqueuse et le contenu en mucus de l’intestin grêle distal est modifié. Les particules provoquent une desquamation cellulaire et des modifications du mucus intestinal. La multiplication virale est achevée en 18 h. Les rotavirus ne peuvent être mis en évidence dans les entérocytes après la phase aigüe de la maladie. La régénération muqueuse n’est pas achevée dans les 4 jours suivant la diarrhée.

MOTS-CLÉS : Rotavirus, Veau, Diarrhée, Intestin ; Muqueuse.

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REFERENCES

[1] Bishop, R. F., Davidson, G. P., Holmes, I. H. & Ruck, B. J., Virus particles in epithelial cells of duodenal mucosa from children with acute non-bacterial gastroenteritis. Lancet, 1973, I, 1281-1283.
[2] Chasey, D., Different particle types in tissue culture and intestinal epithelium infected with rotavirus. J. gen. Virol., 1977, 37, 443-451.
[3] Dubourguier, H. C., Gouet, Ph., Mandard, O., Contrepois, M. & Bachellerie, C., Scanning electron microscopy of abomasum and intestine of gnotoxenic calves infected either with rotavirus, coronavirus or enteropathogenic E. coli. Ann. Rech. vét., 1978, 9, 441-451.
[4] Dubourguier, H. C., Gouet, P., Contrepois, M. & Riou, Y., Élevage de gros animaux axéniques et oligoxéniques en isolateurs. Sci. Tech. Anim. Lab., 1978, 3, 81-85.
[5] Ducluzeau, R., Raibaud, P., Lauvergeon, B., Gouet, P., Riou, Y., Criscelli, C. & Ghnassia, J. C., Immediate postal decontamination as a means of obtaining axenic animals and human infants. Canad. J. Microbiol., 1976, 22, 563-566.
[6] Gouet, P., Contrepois, M., Dubourguier, H. C., Riou, Y., Scherrer, R., Laporte, J., Vautheron, J. F., Cohen, J. & L’Haridon, R., The experimental production of diarrhea in colostrum deprived axenic and gnotoxenic calves with enteropathogenic E. coli, rotavirus, coronavirus and in a combined infection of rotavirus and E. coli. Ann. Rech. vét., 1978, 9, 433-440.
[7] Mebus, C. A., Viral calf enteritis. J. Dairy Sci., 1975, 59, 1175-1177.
[8] Mebus, C. A., Underdahl, N. R. & Rhoades, M. B., Calf diarrhea reproduced with a virus from a field outbreak. Univ. Nebraska Res. Bull., 1969, 233-251.
[9] Mebus, C. A., Stair, E. L., Underdahl, N. R. & Twiehaus, M. J., Pathology of neonatal calf diarrhea induced by a reo-like virus. Vet Path., 1971, 8, 490-505.
[10] Moon, H. W. & Joel, D. D., Epithelial cell migration in the small intestine of sheep and calves. *Amer. J. vet. Res.*, 1974, 36, 187-189.

[11] Newman, L. E. & Trapp, A. L., Lesions of experimentally induced colibacillosis in neonatal gnotobiotic pigs: a scanning electron microscopic study. *Amer. J. vet. Res.*, 1977, 38, 297-305.

[12] Pearson, G. R., McNulty, M. S. & Logan, E. F., Pathological changes in the small intestine of neonatal calves naturally infected with reo-like virus (rotavirus). *Vet. Rec.*, 1978, 102, 454-458.

[13] Riou, Y., Gouet, Ph., Dubourguier, H. C., Contrepois, M., Dardillat, C. & Lefaibre, J., Technique for obtaining, fistulisation and rearing of axenic or gnotoxenique lambs, kids and calves. *Ann. Rech. vét.*, 1977, 8, 13-24.

[14] Scherrer, R., Cohen, J., L’Haridon, R., Feynerol, C. & Fayet, J. C., Reovirus-like agent (rotavirus) associated with neonatal calf gastroenteritis in France. *Ann. Rech. vét.*, 1976, 7, 25-31.

[15] Stair, E. L., Mebus, C. A., Twiehaus, M. J. & Underdahl, N. R., Neonatal calf diarrhea. Electron microscopy of intestine infected with a reovirus-like agent. *Vet. Path.*, 1973, 10, 155-170.

[16] Snodgrass, D. R., Smith, W., Gray, E. W. & Herring, J. A., A rotavirus in lambs with diarrhea. *Res. vet. Sci.*, 1976, 20, 113-114.

[17] Thake, D. C., Moon, H. W. & Lambert, G., Epithelial cell dynamics in transmissible gastroenteritis of neonatal pigs. *Vet. Path.*, 1973, 10, 330-341.

[18] Woode, G. N., Bridger, J. C. & Hall, G. A., The isolation of reovirus-like agents (rotavirus) from acute gastroenteritis of piglets. *J. med. Microbiot.*, 1976, 9, 203-209.