Kinetics, Tissue Specificity and Pathological Changes in Murine Rotavirus Infection of Mice

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SUMMARY

Mice that did not contain antibodies to rotavirus were orally infected with murine rotavirus (EDIM strain) and observed over 7 days. As judged by ELISA, only the small intestine was infected, not the colon. The infection was biphasic, viral antigen peaks being observed at 48 h and approximately 120 h post-infection. Clinically evident diarrhoea was maximal at 72 h. Virus in the upper, middle and lower regions of the small intestine was mainly tissue-associated; most virus was found in the middle small intestine. Two peaks (48 h and 120 h post-infection) of virus antigen were observed in the colon, but these corresponded to luminal, not tissue-associated viral antigen. Only enterocytes in the upper two-thirds of villus epithelia were infected as judged by fluorescent-antibody analysis and transmission electron microscopy. Scanning electron microscopy revealed morphological appearances not hitherto correlated with the progress of the infection: villus tips were convoluted, corresponding to the shedding of virus-infected cells but the lower regions of infected villi were shrunken and considerably narrowed compared to tips.

INTRODUCTION

Advances have been made in our understanding of genetic aspects of the molecular biology of tissue culture-adapted rotavirus (Estes et al., 1983; Cukor & Blacklow, 1984), but despite the importance of rotavirus as a worldwide cause of morbidity and mortality in humans (Kapikian et al., 1980) and animals (Mebus et al., 1971; Snodgrass et al., 1976), little is known about the virus-specific determinants of disease. Data on pathophysiological mechanisms are also few and contradictory.

We have begun to tackle some of these problems using the epizootic diarrhoea of infant mice (EDIM; Cheever & Mueller, 1947) strain of rotavirus and neonatal mice. The disease in mice resembles the self-limiting diarrhoea caused by rotavirus in humans and also provides a potential opportunity for studying the molecular determinants of a disease caused by an animal virus pathogen in its natural host. Several important facts must clearly be established to pursue such objectives meaningfully. These include the clear definition of the pathology and pathophysiology of the infection, and, as described here, establishment of the kinetics and tissue localization of viral replication.

At the commencement of this work, two studies (Coelho et al., 1981; Eydelloth et al., 1984) had been undertaken which were relevant to our projected work, but in themselves constitute an incomplete basis for our purposes. Coelho et al. (1981) investigated EDIM infection of infant mice by light and fluorescence microscopy, scanning and transmission electron microscopy. Histological changes were examined over a period of 10 days post-infection in relation to rotavirus infection; the latter was judged by qualitative scoring of immunofluorescent intensities of frozen sections of different regions of gut sampled over the same period. However,
rotavirus infection was not measured directly by ELISA and the age of the mice used for infection varied from 3 to 5 days, an important point in relation to the rapid maturation of gut tissue in the neonate.

Eydeltoth et al. (1984) used the same system to correlate rotavirus infection (measured by ELISA) with the onset of diarrhoea in seronegative mice of different ages and in immunized animals in attempts to understand the mechanism of immunity to rotavirus infection. However, they did not correlate their data with histological or ultrastructural changes in gut tissue; in addition, their ELISA analyses were performed on whole gut homogenates and not on regional segments of infected gut.

Here, we report experiments in which systematic ultrastructural analyses have been combined with a quantitative ELISA for rotavirus infection. We have confirmed or modified some of the observations of both these groups and defined more critically the anatomical sites and times post-infection necessary for a more detailed study of the pathophysiology and virus determinants of rotavirus-induced diarrhoea in mice. We also highlight hitherto unreported changes in gut structure in response to this virus infection, the correct interpretation of which may be profoundly important in our understanding of the pathophysiology of infectious diarrhoeas.

METHODS

**Virus.** The Cambridge strain of EDIM virus (obtained from Dr T. H. Flewett of the WHO Collaborative Centre for Research on Rotaviruses, East Birmingham Hospital, West Midlands, U.K.) was used in all infection experiments, and was stored at −70 °C.

**Purification of virus.** Crude virus preparations were obtained from previously infected mouse intestines. Whole intestinal tracts taken from two infected mice were homogenized in 100 ml virus suspension buffer (1·5 mM-CaCl₂·2H₂O in 0·1 M-Tris-HCl pH 7·2). The homogenate was then precipitated with 8% polyethylene glycol (M, 6000) followed by 0·5 M-NaCl. Precipitates were collected by centrifugation (10000 g, 30 min, 4 °C), resuspended in 15 ml virus suspension buffer and extracted with an equal volume of trichlorotrifluoroethane (Fluorochem Ltd). The aqueous phase was centrifuged through 45% (w/v) sucrose on to a CsCl cushion. The virus fractions were further purified on a CsCl density gradient as described by Beards (1982). The final preparation was dialysed against 0·1 M-Tris-HCl pH 7·2 containing 1·5 mM-CaCl₂ filtered through a 0·22 μm membrane and stored at −70 °C until required.

The purified virus preparation was tested for the presence of contaminating bacteria by culture on nutrient and blood agar plates at 37 °C. No bacterial growth occurred on plates inoculated with the purified virus preparation. The purified preparation was also examined by transmission electron microscopy. Only virus particles with morphology characteristic of rotaviruses were present.

**Mice.** BALB/c mouse breeding stocks, seronegative for rotavirus, were provided by Dr T. H. Flewett. They were housed for breeding in a positive pressure isolator (Plysu Ltd) and fed gamma-irradiated rodent food. Litters of mice were held in the isolator until required. When 7 days old, they were removed, challenged and held in open cages.

**Infection of mice.** Inocula were derived as follows from 7-day-old EDIM-infected mice killed 48 h post-infection. Infected intestines from five animals were homogenized using a Griffith glass homogenizer in 25 ml virus suspension buffer. This preparation was distributed into 400 μl aliquots and kept at −70 °C until required. Fresh vials were used for each experiment; approximately 30 μl of freshly thawed material was used to infect each mouse orally. This dose contained 10⁸·⁵ ID₅₀; the infectivity of the preparation remained high throughout the period of this work.

All experiments were carried out using 7-day-old animals from (whenever possible) litters of a similar size (about four) to provide animals of comparable maturity. Mice were infected by oral administration of 30 μl of infectious homogenate, returned to their dams and examined three times a day for the presence of faecal staining of abdominal skin. For experiments with purified virus, 30 μl virus suspension (prepared as above) were used.

**Preparation of gut homogenates for ELISA.** In preliminary experiments, individual mice from an infected litter were killed at 24 h intervals. Entire intestinal tracts from duodenum to rectum of killed mice were homogenized in 5·0 ml virus suspension buffer using Griffith tube homogenizers. Homogenates were stored at −20 °C until assayed by ELISA. Both crude and purified inocula were used in this series of experiments.

In subsequent experiments, entire litters infected with crude virus suspension were killed at 24 h intervals post-infection. The intestinal tracts from duodenum to appendix of individual mice were divided into roughly equal thirds and designated upper (U), middle (M) and lower (L) small intestine (SI); the fourth section from appendix to rectum was designated colon (C). Each section was homogenized separately in 1·0 ml virus suspension buffer, using Griffith tube homogenizers. Homogenates were stored at −20 °C until assayed by ELISA.
The distribution of rotavirus antigen between the tissue and lumen of the intestinal regions described above was also investigated. Intestinal segments of infected mice were cut open and luminal contents collected in 1.0 ml virus suspension buffer. Tissue segments were then washed in 20 ml virus suspension buffer and homogenised in 1.0 ml of the same buffer. Rotavirus antigen in serial dilutions of luminal contents and tissue homogenates of each intestinal region was assayed by ELISA.

Preparation of intestinal tissue for transmission electron microscopy (TEM). For logistical reasons, separate litters of mice were used for ultrastructural studies. The intestinal tracts of mice were immersed in fixative (2.5% glutaraldehyde in 0.05 M-phosphate buffer pH 7.4 adjusted to 350 mM with sucrose) within 30 s of decapitation. Full thickness biopsies, approximately 2 cm in length, were taken from U-, M- and L-SI and C. Biopsies were cut open longitudinally and pinned out under fixative. After 1 h of fixation in glutaraldehyde, the biopsies were post-fixed in 1% (w/v) OsO4 in phosphate buffer pH 7.4 made up to 350 mM with sucrose. They were then dehydrated in ethanol and 1 mm² portions were embedded in epoxy-araldite mixture (Mollenhauer, 1964). Sections were stained with methanolic uranyl acetate and lead citrate.

Preparation of intestinal tissue for scanning electron microscopy (SEM). Portions of the same biopsies taken and processed for TEM up to and including dehydration in ethanol (70% v/v) were further treated as follows. Tissues were immersed in 1% (w/v) thiocarbohydrazide for 1 h at 50°C, washed in hot water for 15 min and further treated with 2% (w/v) OsO4 in distilled water for 1 h at 50°C (Ono & Takashio, 1978). Biopsies were then dehydrated via 25, 50 and 100% acetone before critical point drying with liquid CO2. The specimens were fixed to copper plates using electrically conducting paint (RS 555-156). Tissues were examined without coating with metal.

SEM and TEM. Biopsies were examined in both modes with a Jeol 120CX electron microscope, using accelerating voltages of 60 or 80 kV for TEM. SEM images were obtained via a secondary electron detector using accelerating voltages of 20 or 40 kV.

ELISA. Rotavirus antigen was assayed in homogenates of infected intestines by a modification of the WHO rotavirus ELISA method (Beards et al., 1984). Hyperimmune rabbit anti-rotavirus serum was used as the source of coating antibody and hyperimmune guinea-pig anti-rotavirus serum as secondary antibody. Antisera were donated by Dr T. H. Flewett’s laboratory. They had been raised against a combination of purified subgroups 1 and 2 human rotaviruses in rabbits and guinea-pigs. Rabbits received approximately 15 inoculations and guinea-pigs one. These antisera demonstrably cross-react with many strains of non-human rotaviruses including EDIM.

Alkaline phosphatase/rabbit anti-guinea-pig IgG (Miles Laboratories) was used as conjugate. Absorbance values were determined by a Titertek ELISA reader. Blank values were determined by measuring absorbance values generated using uninfected intestine. Tests were regarded as positive when absorbance values were obtained > 3 s.d. above the mean blank value. A positive control sample consisting of a homogenate of an EDIM-infected intestine was included in each plate to correct for variation in ELISA plates. Comparative analyses were made with respect to length of gut (U-, M-, L-SI or C) and not adjusted for mass.

Fluorescent antibody analysis of sections of infected gut. Sections of infected intestinal tissue taken from U-, M-, L-SI and C were fixed in 95% ethanol at 4°C. Sections were then immersed in absolute ethanol at 4°C for three periods of 45 min and subsequently transferred through two changes of chloroform at 4°C. Finally, intestinal sections were wax-embedded, and cut to a thickness of 2 μm. Indirect fluorescent antibody staining was carried out using hyperimmune rabbit anti-rotavirus serum as primary antibody and fluorescein isothiocyanate-labelled sheep anti-rabbit serum (Wellcome) as secondary antibody.

Very little non-specific fluorescence was observed in the small intestine. However, non-specific fluorescence was observed in the colon, but its distribution strongly indicated that this was due to mucus. No attempt was made to remove this by absorption, since TEM analysis gave no indication of the presence of virus in the colon. Only data for the small intestine are recorded.

RESULTS

Infection of mice with crude or purified virus preparations

Since the inoculum used in infection experiments was a crude homogenate of rotavirus-infected mouse intestine, it was possible that symptoms or pathological effects observed in infected animals were the result of a contaminating infectious agent. To eliminate this possibility, a series of experiments was performed investigating the incidence of diarrhoeal symptoms, and the titre of rotavirus antigen in intestinal tracts of mice infected with either crude or purified preparations of EDIM virus was determined. In mice infected with either crude or purified virus preparations, diarrhoeal symptoms first occurred 48 h post-infection, but were most common 72 h post-infection; the attack rate was 100%. Rotavirus antigen levels in the intestinal tracts of infected mice were maximal at 48 h post-infection. Based on these data, all subsequent experiments were carried out using crude preparations of EDIM.
Fig. 1. Kinetics of EDIM virus antigen production in neonatal mouse intestine. Mice were orally infected with EDIM virus. At 24 h intervals post-infection, animals were killed and titres of EDIM virus antigen in homogenates of (a) upper, (b) middle and (c) lower small intestine, and (d) colon were determined by ELISA. Vertical bars indicate s.e.m.; n = 4 at 24, 96, 144 and 168 h and 8 at 48, 72 and 120 h.

Rotavirus antigen levels in combined tissue and luminal contents of mouse intestine

Rotavirus antigen levels in U-, M-, L-SI and C of EDIM-infected mice at times up to 168 h post-infection were determined by ELISA (Fig. 1). In all regions of the intestine studied, maximal levels of rotavirus antigen occurred 48 h post-infection. At later stages of infection, between 48 and 144 h post-infection, a second peak was found. Antigen levels, representing the sum of tissue and luminal antigen, were greatest in the L-SI and C (Fig. 1c, d respectively).

Distribution of rotavirus antigen in tissue and lumen of mouse intestine

Maximal tissue levels of rotavirus antigen occurred 48 h post-infection in all regions of the intestinal tract studied. In the later stages of infection, antigen titres declined, then subsequently became elevated. M- and L-SI were most heavily infected; lower antigen levels were present in the U-SI and C. Luminal antigen levels were low in all regions of the SI throughout the infection, whereas in colon, high rotavirus antigen levels occurred at times coincident with the appearance of the two peaks of tissue antigen detected in the SI (Fig. 2).

Immunofluorescent analysis of rotavirus-infected small intestine

The distribution of rotavirus antigen in different regions of small intestine at times up to 168 h correlated well with ELISA data in Fig. 1 and 2. Rotavirus antigen levels at all times were highest in lower small intestine, followed by middle then upper small intestine. In all antigen-containing regions, the maximum level of rotavirus antigen occurred at 48 h post-infection. Antigen was present in epithelial cells on the upper two-thirds of intestinal villi with no evidence of infection in the lower one-third or in the crypts (Fig. 3). By 96 h post-infection, extensive vacuolation of enterocytes had occurred, but no rotavirus antigen was evident within these vacuoles. No antigen was detected after 144 h post-infection.

SEM analysis of rotavirus-infected intestine

Fig. 4(a) shows the typical SEM appearance of control mouse M-SI at 9 days of age. Villi were smooth and regular with parallel or cone-shaped morphology, but considerable variation in the length of villi was observed. At 48 h post-infection, rotavirus-infected M-SI showed abnormal villus architecture. The middle to base regions were shrunken and puckered, with tips being relatively swollen, but of the same order of width as controls (Fig. 4b). Tips exhibited a
Fig. 2. Distribution of viral antigen between tissue and lumen of EDIM virus-infected neonatal mouse intestine. At 24 h intervals after infection with EDIM virus, animals were killed and titres of EDIM virus antigen in the tissue (●) and lumen (○) of (a) upper, (b) middle and (c) lower small intestine, and (d) colon were determined by ELISA. Vertical bars indicate S.E.M. (n = 4).

Fig. 3. Typical distribution of viral antigen by fluorescent antibody analysis of EDIM virus-infected mouse small intestine. (a) Control; (b) 48 h post-infection. Note localized distribution of fluorescence in epithelial cells on upper two-thirds of infected villus.

corroded appearance corresponding to shedding of large numbers of enterocytes (see TEM, Fig. 5). The deformation of villus structure was observed throughout the infective process, although considerably fewer enterocytes were observed in the process of shedding from villus tips in the latter stages of infection. Similar, but less marked changes were observed in U- and L-SI. Data for U-, L-SI and C are not shown.
Fig. 4. Scanning electron microscopy of (a) control middle small intestine and (b) middle small intestine 48 h after infection with EDIM virus. Note the finger or cone-shaped normal villi with regular smooth surface appearance (a). In contrast, note the appearance of infected villus tips (medium and large arrows) corresponding to infected enterocytes being shed (see Fig. 5b), and shrunken (small arrows) appearance of middle to base regions (b). Bar markers represent 100 μm.
Fig. 5. Transmission electron micrograph of full-length villi from control and EDIM virus-infected mice. (a) Control showing uniform staining and peripheral pinocytotic vacuoles; (b) infected villi from middle small intestine at 48 h post-infection with tips (arrows) having a morphology similar to those identified by like arrows in Fig. 4(b). Note the differential electron densities exhibited by infected villi, the shrinkage of middle to base regions, irregular vacuolation within and between enterocytes and oedema in lamina propria (left villus). (c, d) Detection of virus particles in vacuolated regions; (c) magnification of the small boxed area in (b). Bar markers represent 20 μm for (a, b), 1 μm for (c) and 0.2 μm for (d).
Fig. 5 shows the typical TEM appearance of mouse M-SI. Full-length, or near full-length, sections were examined at very low magnification. This required large area sections of a thickness greater than is normal for high resolution TEM. Such sections proved invaluable in determining the presence and distribution of virus particles within individual villi and relating such to the overall response of infected villi. The micrographs were typical, but were selected on the basis of being near central, longitudinal sections through the lamina propria. The following observations were made.

(i) The sectional profile size and shape of villi as observed in TEM were as would be predicted by SEM. In particular, one can be confident in interpreting the convoluted appearance in SEM as evidence of cell shedding. Villi may have been shortened slightly, but were definitely shrunken towards their bases and more closely packed.

(ii) Virus was detected (see Fig. 5c, d) in enterocytes along two-thirds of villi from the tip downwards, confirming the fluorescent antibody analysis (Fig. 3). None was seen in crypt regions.

(iii) Vacuolation observed in control tissue comprised spherical supranuclear pinocytotic vesicles, a characteristic of the neonatal gut (Clark, 1959). In contrast, infected tissues showed more-pronounced vacuolation of the enterocytes. This vacuolation was more widespread throughout these cells, occupying all regions of the cytoplasm. Moreover, some enterocytes had vacuolated nuclei. Enterocytes were also shrunken laterally, leaving enlarged intercellular spaces (Fig. 5b).

(iv) More-detailed analysis showed that the mucosal brush border remained largely intact, but that enterocytes (in particular those below the area of irregular vacuolation) were shorter along their vertical trans-epithelial axis. Lamina propria consistently appeared more electron-dense, possibly suggesting (although not formally proving) that the fluid surrounding lamina propria in the large irregular vacuoles was hypertonic. This was not an occasional artefact, since in the lower regions of infected villi (Fig. 5b) the electron density of enterocytes was comparable to that of control enterocytes (Fig. 5a). In three villi (Fig. 5b), elements of lamina propria and occasional enterocytes showed this increased electron density. No massive ulceration was seen in the sense of extensive stripping of enterocytes leaving exposed lamina propria. Cells were shed mainly from the tip regions of villi by a process that led to concomitant resealing or recovering of lamina propria.

**DISCUSSION**

Rotavirus antibody-free mice are highly susceptible to challenge with EDIM virus. An attack rate of 100% occurred in mice challenged with either crude or purified virus preparations; the kinetics of infection were also similar. This suggests that symptoms and pathological effects observed in infected mice were not due to the presence of a contaminating infectious agent. The high number of infectious doses per animal used in this work is in line with current work (Eydelloth et al., 1984) and clearly ensured a highly reproducible pattern of infection. The justifiable assumption is that only existing susceptible gut cells are infected and that virus in excess of this requirement is simply extruded; it was not possible to detect virus antigen by ELISA up to 24 h post-infection, even with the high infectious dose of virus used.

A marked increase in rotavirus antigen titre was observed in all segments of small intestine between 24 and 48 h post-infection. This coincided with the onset of clinical diarrhoea and with the first appearance of mature rotavirus particles. Investigation of the distribution of rotavirus antigen between intestinal tissue and lumen revealed that antigen titres in the colon were mostly luminal and thus presumably secondary to virus replication in the small intestine. It is possible that the low levels of rotavirus antigen detected in colon tissue were due to the presence of antigen adhering to mucus on the tissue surface. Failure to observe rotavirus particles in TEM preparations of colon tissue lends support to this hypothesis. These findings are in agreement with the results of Adams & Kraft (1967) and Coelho et al. (1981), but conflict with Banfield et al. (1968), who reported that virus particles were present in all regions of the intestine distal to the stomach.
Gouvea et al. (1986) recently published a study of human rotavirus (strain MET) infection in mice. Only few comparisons are possible with the work presented here. The pathology of the infection was examined by haematoxylin and eosin staining of duodenum sections only at three time points; vacuolation in the upper regions of villi was seen. Their limited SEM data are difficult to interpret. They showed no evidence of virus replication as judged by an increase in tissue culture infectious doses in recovered tissues over the original inoculum. Their kinetic data were obtained on whole gut (excluding duodenum) homogenates and show one single point increase (12 h after inoculation) in a generally declining virus infectivity curve. These authors could not exclude the possibility of a 'toxic viral action'.

The pathology of rotavirus infection has been studied in other animal species, including gnotobiotic calves (Mebus & Newman, 1977), lambs (Snodgrass et al., 1977) and piglets (Theil et al., 1978). Rotavirus infection in these species, in common with the murine rotavirus infection described in this paper, exhibits tropism for enterocytes lining the upper regions of intestinal villi. However, the pathology of infection in these species differs in certain respects from EDIM infection of mice. A blunting of villi has been reported in rotavirus infection of calves (Mebus & Newman, 1977), lambs (Snodgrass et al., 1977) and piglets (Theil et al., 1978). This effect takes an extreme form in rotavirus infection of piglets, where complete jejunal villus erosion has been described (Theil et al., 1978). No significant reduction in length of villi accompanied the murine rotavirus infection in the present study. Additionally, the extensive intra- and intercellular vacuolation reported here has not been highlighted as a major feature of rotavirus-induced pathology in calves (Mebus & Newman, 1977), lambs (Snodgrass et al., 1977) and piglets (Theil et al., 1978). In these species, denudation of the upper regions of affected villi occurred, leaving the lamina propria exposed; this type of pathology was not observed in this study.

The kinetics of rotavirus infection in gnotobiotic piglets has been investigated by Crouch & Woode (1978), who used an immunofluorescent infectivity assay to determine viral titres in luminal contents taken from different intestinal regions of infected animals for times up to 21 days post-infection. A biphasic pattern of infection and recovery from infection was detected in this study; the authors claimed that non-immune mechanisms were responsible for the initial reduction in virus titre, and that the secondary decline was due to the effect of antibody. No experimental evidence, however, was presented in support of this hypothesis.

The biphasic pattern of rotavirus antigen production found in the present study is similar to that described by Eydelloth et al. (1984). The drop in virus antigen titre at 72 h could be a result of the shedding and accelerated clearance of viral antigen associated with commencement of diarrhoeal symptoms in infected animals and temporary loss of cells with specific receptors necessary for infection. The second peak could result from infection of replacement cells generated in crypts and which bear surface receptors for the virus.

Thus, our results identify stages in the infective process, and regions of the intestinal tract which will be the subject of future pathophysiological investigations. Clearly, events occurring between 24 and 48 h post-infection must be involved in initiating diarrhoea which reached a peak about 72 h post-infection. To resolve the virological aspects of this phenomenon, a biochemical analysis of rotavirus enterocyte interactions will be required either in vivo or in a relevant in vitro system as attempted by Lees & Stephen (1985) for ectromelia virus and primary murine hepatocytes. From a pathophysiological standpoint, one must seek to interpret the response of villi and not just enterocytes to infection by rotavirus since, despite the fact that infection by virus was restricted to apical and lateral enterocytes, the entire length of villi were affected in the manner described in Fig. 4 and 5; bases were clearly shrunken. The origin and nature of these villus changes is being actively pursued.

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