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Reovirus infection in adult mice: the virus hemagglutinin determines the site of intestinal disease

Donald H. Rubin,[1]* Martha A. Eaton[1] and Arthur O. Anderson[2]

[1] *Departments of Medicine and Microbiology, University of Pennsylvania, Philadelphia, PA 19104, U.S.A.;* [2] *U.S.A. Medical Research Institute of Infectious Disease, Fort Detrick, Fredrick, Maryland, U.S.A.*

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Reovirus type 1, strain Lang, and type 3, strain Dearing, induced site-specific intestinal lesions in the adult mouse after intravenous inoculation. Reovirus type 1 caused inflammation and epithelial changes such as loss of nuclear polarity, villus blunting and crypt hyperplasia restricted to the ileum. In contrast, reovirus type 3 induced duodenitis, jejunitis, and ulcerative colitis. In the duodenum and jejunum, the epithelial cells appeared normal, but hemorrhage and inflammation in the lamina propria was present. In the colon, superficial ulceration, crypt abscesses, and intraluminal hemorrhage was observed. Segregation analysis using reassorant clones derived from reoviruses 1 and 3, suggested the viral hemagglutinin, encoded by genome segment S1, to be the major viral determinant of site specific intestinal disease following intravenous inoculation.

Key words: reovirus; duodenitis; jejunitis; ileitis; ulcerative colitis; viral hemagglutinin.

Introduction

The gastrointestinal (GI) tract is the portal of entry of several viruses which produce systemic disease, including enteroviruses, coronaviruses, and reoviruses. Reoviruses have been used in a mouse model to examine the GI phase of infection.

In neonatal mice infected perorally with reovirus type 1, viral titers in gut associated tissues increase approximately 10-fold during the first 3 days of infection.[1] This increase in titer in the GI tract is considerably less than that obtained with *in vitro* reovirus infection of L cells, or with infection of viruses (such as rotaviruses) that are known to replicate within epithelial cells located throughout the GI tract.[2,3] Since a large increase in titer was not observed, these data suggest that the sites of replication of reovirus type 1 in the intestine may be limited.

Wolf and co-workers[4,5] examined the fate of input virus by inoculating reovirus type 1 into closed loops of ileum and performing electronmicroscopic examination of the infected ileum 5 minutes to 24 hours following infection. They found that reovirus type 1 is adsorbed to microfold (M) cells and enters the host specifically through these cells which are associated with Peyer's patches in both suckling and adult mice.

To examine infection in the adult intestine, high doses of reovirus type 1 were used.[6] When 10^{10} plaque forming units (pfu) of reovirus type 1 were inoculated either perorally or intraduodenally, virus was found to replicate in epithelial cells located in the crypts of Lieberkuhn and replication was restricted to the ileum.[6]

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Based upon the specific site of adsorption and limited region of replication of reovirus type 1 in the intestine, we suggested that virus entered the host via M cells overlying Peyer's patches and then infected the crypt cells after adsorption to the basolateral membranes of crypt cells. We further hypothesized that a systemic route of reovirus type 1 administration should result in infection of crypt cells of the ileum due to specific receptors on these cells. In the following studies, we determined that reovirus type 1 produces perforation and/or villus blunting and crypt hyperplasia of the ileum by both intravenous and peroral routes of virus administration, consistent with our hypothesis.

Little is known about the exact nature of GI infection due to other reovirus serotypes. In the newborn mouse, peroral administration of reovirus serotype 3 results in approximately a 100-fold decrease in virus titers during the first 3 days of infection.[1] In the suckling mouse, Wolf and colleagues found that reovirus type 3 absorbs to intestinal epithelial cells in the ileum, but does not replicate within these cells.[5] Thus, the site of virus replication or disease induction within the intestine have not been determined for reovirus type 3.

Results

Intravenous inoculation of reovirus type 1 induces ileitis

Previous studies[6] with reovirus type 1 have demonstrated that, after peroral infection of A/J mice, virus replication was restricted to the ileum. The purpose of this study was to determine whether intravenous inoculation would result in pathologic changes similar to those seen after oral inoculation. After intravenous inoculation of 10^{10} PFU of reovirus type 1, all mice appeared acutely ill by 36 hours, and all died by 48 hours. Autopsy of the moribund mice showed marked inflammation in the lamina propria of the ileum associated with hyperplasia of the crypts and foreshortening of the villi (Fig. 1). Thus,

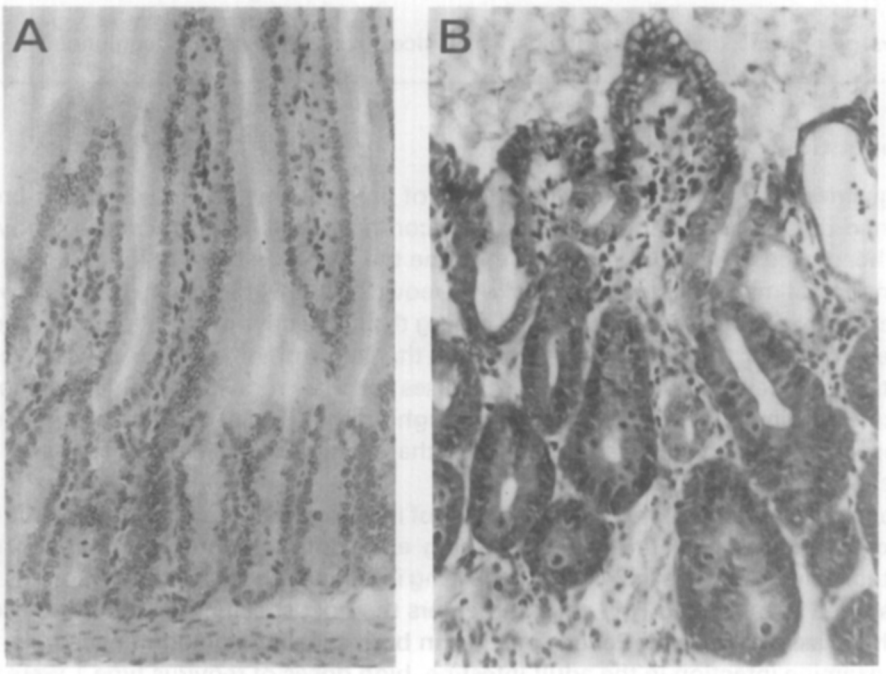

Fig. 1. Reovirus type 1-induced lesions in the ileum. (a) Normal ileum (150 ×). (b) Representative lesions in adult A/J mice inoculated intravenously or perorally with 10^{10} PFU of reovirus type 1. There is evidence of villus blunting and marked crypt hyperplasia with lack of nuclear polarity in the epithelial cells, and an inflammatory infiltrate in the lamina propria (150 ×).

Table 1 Reovirus titers in intestinal contents of A/J mice 24 hours after infection[a]

Intestinal segment	Reovirus serotype or reassortant clone			
	1	3	3.HA-1	1.HA-3
Duodenum	3.45±0.49[b]	<1	3.26±0.837	3.51±0.541
Jejunum	2.68±2.19	<1	2.26±1.87	0.90±1.80
Ileum	2.86±1.63	<1	3.17±0.316	1.35±1.56
Colon	2.26±1.86	<1	1.49±0.211	<1

[a] Mice were inoculated intravenously with 10^{10} PFU of reovirus type 1, type 3, or reassortant clones, and sacrificed 24 hours following infection.
[b] Titers are expressed as $\log_{10}$ PFU per ml of luminal contents ± standard deviation.

reovirus type 1 induced similar pathologic changes after either intravenous or peroral inoculation. In addition to the pathologic changes in the ileum, there was evidence of extensive pneumonitis and focal glomerulitis (data not shown).

At 24 hours after infection with reovirus type 1, infectious virus could be recovered from the intestinal contents as well as from all segments of the bowel (Tables 1, 2). Thus, infectious reovirus type 1 was widely distributed in the intestine at 24 hours after infection, yet at 48 hours after infection microscopic evidence of disease was limited to the ileum.

Intestinal disease due to reovirus type 3 requires intravenous inoculation

Previous studies have not shown any evidence of viral replication in the intestine after peroral inoculation of this virus.[1,5] We inoculated mice perorally with 10^6 to 10^{10} PFU and no microscopic evidence of GI pathology was seen even at the highest dose. Similarly, intravenous inoculation with 10^8 and 10^9 PFU per mouse of reovirus type 3 produced no evidence of disease. However, mice inoculated intravenously with 1×10^{10} PFU per mouse appeared ill at 24 hours with evidence of diarrhoea and dehydration, and 7 of 7 mice died by the second day following inoculation (Table 3).

The intestines of mice receiving an intravenous inoculum of 10^{10} PFU showed hemorrhagic areas in the duodenum, and jejunum at 48 hours after infection. Histologic examination revealed hemorrhage in the lamina propria of the duodenum (Fig. 2), and a leukocyte infiltrate was observed in the jejunum. Microscopic examination of

Table 2 Reovirus titer in intestinal tissue of A/J mice 24 hours after infection[a]

Intestinal segment	Reovirus serotype or reassortant clone			
	1	3	3.HA-1	1.HA-3
Duodenum	5.42±0.66[b]	0.849±0.12	3.71±0.46	3.26±0.837
Jejunum	4.93±0.26	<1	2.92±0.11	2.29±0.061
Ileum	4.53±0.14	0.20±0.352	3.47±1.38	3.17±0.316
Colon	3.53±1.55	3.10±0.98	2.94±2.59	1.49±2.11

[a] Mice were inoculated intravenously with 10^{10} PFU of reovirus type 1, type 3, or reassortant clones, and sacrificed 24 hours following infection.
[b] Titers are expressed as $\log_{10}$ PFU per mg of intestinal tissue ± standard deviation.

Table 3 Effect of the initial inoculum of reovirus type 3 on pathology[a]

Inoculum	Colitis[b]	Diarrhoea[c]	Death[c]
1×10^{10}	+ + + +	7/7	7/7
5×10^{9}	+ + +	5/10	0/10
1×10^{9}	+ +	2/10	0/10
1×10^{8}	+	0/10	0/10

[a] Adult A/J mice were inoculated intravenously with various doses of reovirus type 3 (Dearing).

[b] Mice were sacrificed on the second day after inoculation and specimens were examined for gross and microscopic pathologic lesions. Pathology was scored in the following manner: + + + +, hemorrhage into the lumen of intestine, crypt abscesses, inflammatory cell infiltrate in the lamina propria, mucus depletion, epithelial cell irregularity, goblet cell hypertrophy; + + +, inflammatory cell infiltrate in the lamina propria, mucus depletion, epithelial cell irregularity, goblet cell hypertrophy; + +, inflammatory cell infiltrate with either mucus depletion, epithelial cell irregularity, or goblet cell hypertrophy; +, inflammatory cell infiltrate with goblet cell hypertrophy.

[c] Mice were observed for seven days for evidence of diarrhoea or for mortality. Diarrhoea was scored as the ability to express a liquid or semi-formed stool.

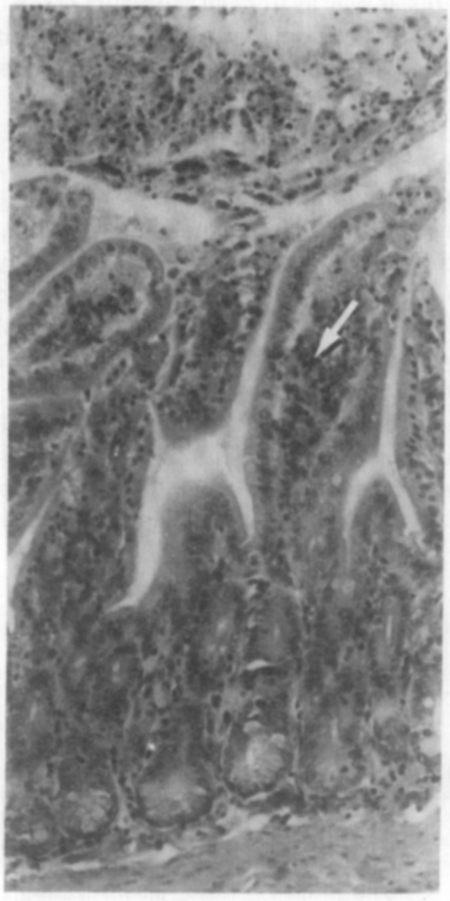

Fig. 2. Reovirus type 3 induced lesions of the duodenum. Reovirus type 3, 10^{10} PFU, was inoculated intravenously into adult A/J mice. There is an inflammatory infiltrate consisting of polymorphonuclear leukocytes and lymphocytes in the lamina propria and hemorrhage without perforation of the intestine (arrow). The epithelial cells appear normal (150×).

the colon revealed superficial ulceration and hemorrhage, with polymorphonuclear leukocytes surrounding the crypts and at areas of ulceration (Fig. 3). In those areas of the colon without hemorrhage, mucosa was depleted of mucus secretion and had irregular glandular contours. In summary, high-dose intravenous inoculation of reovirus type 3 resulted in disease in the duodenum and jejunum, with severe ulcerating lesions confined to the proximal colon. Microscopic examination of other organs revealed that there was only focal pneumonitis.

To determine the sites of GI infection in mice inoculated intravenously with reovirus type 3, virus was assayed from all intestinal segments (Table 1). No infectious virus was detected in the saline washes of the lumen from any intestinal segment, whereas reovirus type 3 was obtained in low titer associated with cells from all sections of intestine (Table 2). The highest viral titers were recovered from the duodenum and colon.

Mild colitis is induced by UV inactivated reovirus type 3

Since infectious virus was not evident within the intestinal lumen of mice inoculated intravenously with 10^{10} PFU of reovirus type 3, we wanted to determine whether replicating virus was essential for the development of colitis. Six mice were inoculated intravenously with 10^{12} particles per mouse of UV-inactivated virus, equivalent to 10^{10} PFU. None of the mice appeared ill at any time following inoculation, and stools

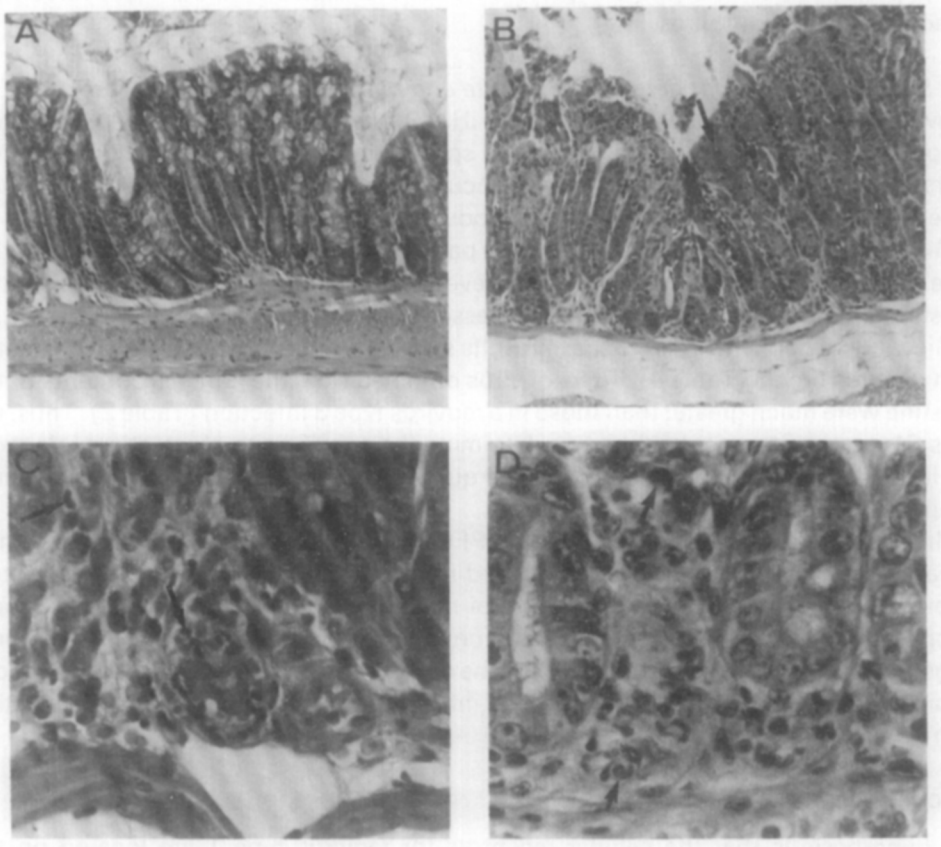

Fig. 3. Reovirus type 3 induced lesions in the colon. Adult A/J mice were inoculated intravenously with 10^{10} PFU of reovirus type 3 and sacrificed two days later. (a) Normal colon (105 ×). (b) Superficial ulceration and hemorrhage (arrow). (105 ×). (c) Inflammation of lamina propria and inflammatory cells in crypt-associated venules (420 ×). (d) Crypt abscesses (arrow) and inflammation of lamina propria (420 ×).

Table 4 The S1 gene determines the sites of intestinal pathology in adult A/J mice inoculated intravenously[a]

Parent or reassortant clone	Parental origin of genome segments										Disease	
	Outer capsid[b]			Core[b]					NS[b]			
	M2	S1	S4	L1	L2	L3	M1	S2	M3	S3	Colitis	Ileitis
1	1	1	1	1	1	1	1	1	1	1	−	+
3.HA-1	3	1	3	3	3	3	3	3	3	3	−	+
3	3	3	3	3	3	3	3	3	3	3	+	−
1.HA-3	1	3	1	1	1	1	1	1	1	1	+	−

[a] Mice were inoculated intravenously with 10^{10} PFU of reovirus parents or reassortant clones. Two days after inoculation, mice were sacrificed and the bowel was examined for pathologic changes. The presence of disease is indicated by a (+).

[b] Physical location of viral polypeptides encoded by each genome segment. NS: non-structural proteins are encoded by these genome segments.

appeared normal. However on day 2, light microscopic examination of the colon revealed that three of the six mice had evidence of a mild inflammatory cell infiltrate, a 2-fold increase in mitotic activity, and distended appearing goblet cells. Virus plaque assays performed on intestinal samples from mice inoculated with UV-inactivated virus detected no infectious virus.

The S1 genome segment determines the site and pattern of intestinal disease

Reovirus reassortant clones 3.HA-1 and 1.HA-3 were used to determine which viral genome segment encodes viral tropism to specific regions of the intestine (Table 4). Within 24 hours following intravenous inoculation with 10^9 pfu of either clone, mice appeared moribund, but the two clones produced different diseases.

Mice inoculated with 3.HA-1 presented a pattern of disease similar to that produced in mice infected with reovirus type 1. However, titers obtained from the segments of intestine showed that 3.HA-1 appeared in lesser amounts than reovirus type 1 (Table 2).

Mice inoculated with 1.HA-3, gave results similar to those observed in mice infected with reovirus type 3 (Table 4). Titers of virus obtained from the lumenal contents of the intestine were much greater than those in a reovirus type 3 infection (Table 2). Thus, the virus S1 genome segment appears to determine the specific site of intestinal infection, but it does not completely account for the quantity of infectious virus in the intestinal samples.

Mice inoculated intravenously with 10^{10} PFU of clone 1.HA-3 had a similar pattern of disease to that observed in mice inoculated intravenously with reovirus type 3. Titers obtained from segments of intestine and of mice inoculated with clone 1.HA-3 were comparable to titers obtained after inoculation of clone 3.HA-1 (Table 2). Thus, the virus S1 genome segment appears to determine the quality of disease in the intestine, but does not account completely for the presence of infectious virus in the lumen of the intestine or in other organs.

Discussion

Our hypothesis is that the cellular receptors for reovirus type 1 are located on the basolateral membrane of susceptible epithelial cells in the ileum.[6] In support of this, mice infected intravenously with reovirus type 1 developed GI disease only in the ileum. In addition, the importance of specific cellular receptors for virus is supported by the results

of experiments using the reassortant clones, 3.HA-1 and 1.HA-3. By segregation analysis, the S1 genome segment, encoding the viral attachment polypeptide (hemagglutinin) is the major viral determinant of the site of intestinal pathology. Thus, reovirus type 1 infects epithelial cells in the ileum due to the presence of specific receptors on the susceptible cells.

However, reovirus type 1 is also found within the lumen of the intestine 24 hours after intravenous administration. This finding raises the possibility that infection is mediated by cellular receptors for the virus on the luminal surface of the epithelial cells. This is unlikely due to the rapidity with which disease develops in the ileum after intravenous administration (<48 hours) compared to peroral administration (>96 hours).[6] We propose that histologic evidence of pathology in the ileum is dependent upon a critical number of cells infected by reovirus type 1, and that susceptible cells are more likely to come into contact with virus administered systemically than perorally. Thus, disease was induced earlier after systemic rather than intraluminal virus exposure. Furthermore, the delay in onset of pathologic changes in the ileum after virus is administered by the peroral route would indicate that the limited number of M cells, found only over Peyer's patches, provide a barrier to rapid infection of susceptible epithelial cells containing viral receptors located on the basolateral membrane.

We are uncertain why reovirus type 1 was recovered from sections of bowel in which there was no evidence of pathology. However, this finding may indicate infection or uptake of virus in macrophages or NK cells associated with the lamina propria. This is consistent with the finding of Letvin *et al.*[7]

Our model of reovirus infection in the intestine predicts that after systemic administration other reovirus serotypes would be capable of inducing GI disease due to receptors for virus on the basolateral membrane of susceptible or specific epithelial cells. Peroral inoculation with reovirus type 3 did not induce pathologic changes nor disease at any site in the adult mouse. This finding is consistent with our hypothesis that specific receptors are required for infection to occur. In neonatal mice, peroral inoculation of reovirus type 3 results in low viral titers in the intestine, and lethal encephalitis does not develop.[1] In suckling mice, Wolf and colleagues demonstrated that reovirus type 3 absorbs to and enters epithelial cells in the ileum, but does not replicate.[5] Our experiments support the finding of Wolf *et al.* that uptake of virus on the luminal surface of epithelial cells does not result in a productive infection.

There are several unusual features of reovirus type 3 GI disease induced by intravenous inoculation: (i) virus was not detectable in the saline washes of the intestinal lumen from any segment of bowel at any time; (ii) immunoperoxidase staining for viral antigen failed to identify any antigen-positive epithelial cells (data not shown);[6] and (iii) extremely high doses of virus were necessary to produce disease. These findings may indicate that reovirus type 3 has an abortive replicative cycle in the intestinal epithelial cells of the duodenum, jejunum, and colon. In support of this interpretation are the findings that UV-inactivated virus induced mild intestinal pathologic changes compared to the severe disease induced by equivalent inoculums of live virus. Thus, the capacity of UV-inactivated virus to cause pathologic changes in the colon may be due to a toxic effect of some virion associated polypeptides in the inoculum on cellular metabolism.[8,9] The more severe pathology obtained with live reovirus type 3 inoculation suggests that there was some degree of replication or abortive infection. The lack of both antigen positive epithelial cells and infectious virus in the intestinal lumen may indicate that the primary site of infection of reovirus type 3 is either endothelial cells of the capillary plexus, or local parasympathetic nerve cells. Therefore, GI disease due to reovirus serotypes other than type 1 may be due to infection of other target cells than epithelial cells infected by reovirus type 1.

Although viral hemagglutinin is the major determinant for development of site-specific disease, the ability to recover infectious virus in the intestine may also be influenced by other genes. Reassortant clone 1.HA-3, which contains the S1 gene from reovirus type 3, was present in higher titer than reovirus type 3 in both segments of intestine and the intestinal lumen. Variance from the anticipated pattern of virus recovery in the lumen of the intestine was also encountered when mice were infected with reassortant clone 3.HA-1, containing the S1 gene of reovirus type 1. Titers in the intestine were somewhat lower than with the reovirus type 1 parent. Thus, S1 gene function does not totally account for the virus recovery in the lumen. Whether the entry of virus into the lumen of the intestine in adult mice is the property of more than one viral gene is currently under investigation.

If our hypothesis on the replicative strategy of reoviruses reflects a general model of GI disease induced by other viruses, then it would suggest that: (i) fecal examination for a viral agent as an etiology of bowel pathology may be fruitless (reovirus type 3), or (ii) an extensive pathologic survey may be required to locate viral disease (reovirus type 1). Whether reovirus infection can provide a model for other GI pathogens (such as Norwalk-like virus) or disease processes (such as ulcerative colitis) is being explored.

Materials and methods

Mice

Adult female A/J mice (Jackson Laboratories, Bar Harbor, ME), 8–12 weeks old, were fed a house diet *ad libitum* (Purina, St. Louis MO). There was no evidence of virus specific antibody in any mouse used for these experiments.

Virus

Reovirus type 1 (Lang), reovirus type 3 (Dearing), and recombinant clones derived from serotypes 1 and 3 have previously been described.[2,10,11] The genome composition of the recombinant clones is shown in Table 4. For mouse inoculation, a stock of reovirus that was passed twice in L-cells was purified by substituting ultrasonic disruption (Branson Ultrasonic 200) for cell homogenization in a modification of published techniques.[12] The particle to PFU ratio was 100:1 for purified virus stocks used in these experiments. Reovirus was inactivated by placing virus 14 cm from a UV source for 15 minutes.[13] No infectious virus was found when UV-treated virus was titered on L-cells.

Mouse inoculation

Groups of 4–7 mice were inoculated i.v. via the tail vein with either 5×10^9 or 10^{10} of reovirus type 1, reovirus type 3, or recombinant clones suspended in 0.2 ml sterile saline containing gelatin. Other groups of mice were inoculated perorally with 10^6, 10^8, 10^9, or 10^{10} PFU of reovirus type 3 by passing a fine catheter (PE-50 polyethylene tubing, Clay Adams, Parsipanny, NJ) through the mouth and into the stomach.[14] Mice were observed for 10 days following inoculation for evidence of diarrhoea or systemic illness.

Titration of virus from mouse tissue

Mice were sacrificed and organs were collected for virus titration or pathology. The small intestine, from the gastroduodenal junction to the ileocecal valve, was removed and divided into proximal, middle, and distal segments approximating duodenum, jejunum, and ileum. The large intestine consisting of the cecum and colon was assayed for virus. The lumen of each bowel segment was washed with 1 ml of saline/gelatin which was collected into sample vials and the segment was then placed into 1 ml of saline containing gelatin. All samples of intestinal contents were frozen and thawed three

times. Tissue samples were disrupted by ultrasound (Branson Ultrasonic 200), and then assayed on L-cell monolayers in 12-well cluster plates (Costar).[6] Direct comparisons between the titers of virus obtained from the intestinal contents and tissues is not possible due to the necessity to dilute the contents of the lumen in the process of collection and the lack of a measurable quantity of protein in the luminal contents.

Pathologic specimens

All specimens were fixed in Bouin's solution for routine histology and immunocytochemistry. Immunoperoxidase staining was performed as previously described.[6]

Protein concentration of intestinal specimens

The concentration of protein in specimens of intestine was determined by the Lowry method.[15]

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