MOLECULAR BASIS OF REOVIRUS VIRULENCE

Role of the M2 Gene*

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The upper alimentary tract is the portal of entry for enteric viruses. After ingestion, viruses may infect cells in the throat, the stomach, or the small intestine. Those viruses that multiply primarily in the small intestine, such as the human rotaviruses, polioviruses, Coxsackie, ECHO virus, hepatitis A, and Norwalk agent (1, 2), come into direct contact with intestinal fluids consisting mainly of acids, proteolytic enzymes, and bile. Enteroviruses, such as Coxsackie, ECHO virus, and polioviruses, are known to be resistant to bile salts and acid (2, 3). Viral resistance to intestinal contents is clearly important if infection is to succeed.

The mammalian reoviruses serotypes 1, 2, and 3 have been isolated primarily from the feces and are thus enteric viruses (4, 5). They are known to be resistant to inactivation by bile salts as well as acid ([2]; and D. Drayna. Personal communication.). A number of investigators have examined the response of reoviruses to digestion by proteolytic enzymes in vitro. McClain et al. (6) and Spendlove et al. (7, 8) initially reported that the infectivity of different mammalian reoviruses could be enhanced or depressed after treatment with chymotrypsin. Subsequently Joklik (9) found that treatment of reovirus with chymotrypsin degraded the outer capsid polypeptides via a series of steps. The precise pattern of degradation was dependent upon virus concentration, concentration of chymotrypsin, and ionic strength of the suspending medium. Shatkin and La Fiandra (10) isolated partially uncoated infectious subviral particles after digestion of purified reovirus with chymotrypsin and noted that 0.14 M NaCl present in the reaction mixture protected reovirus against loss of infectivity. Borsa et al. (11–13) have suggested that there are two discrete stages of uncoating: the first stage is proteolytically mediated and generates infectious subviral particles, and the second stage is mediated by an endogenous mechanism that is triggered by intracellular concentrations of K^+ ions and results in more complete uncoating, loss of infectivity, and transcriptase activation. Although the precise biochemical steps are not completely understood, Joklik (9) has found that cleavage of μ1C (previously termed μ2) is associated with loss of infectivity and activation of transcription.

Reovirions contain 10 segments of double-stranded RNA (dsRNA).[1] These segments

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[1] *Abbreviations used in this paper:* BTEE benzoyl-L-tyrosine-ethyl ester; CHT, chymotrypsin; dsRNA, double-stranded RNA; pfu, plaque-forming units.

are named according to size classes: three large segments (L1, L2, L3), three medium segments (M1, M2, M3), and four small segments (S1, S2, S3, S4). In the virion, the dsRNA is surrounded by a double capsid: an internal core that contains the viral transcriptase and an outer capsid (14). The outer capsid contains three polypeptides, μ1C (derived by cleavage from the primary μ1 polypeptide), σ1, and σ3 that are encoded by dsRNA genome segments M2, S1, and S4, respectively (15–18). Using hybrid recombinant clones consisting of genome segments derived from serotype 1, strain Lang, or serotype 3, strain Dearing, we have shown previously that the σ1 polypeptide is the viral hemagglutinin (15). The σ1 polypeptide, in addition, serves as the type-specific neutralization antigen (19), determines cell tropism (20), and binds to cellular microtubules (21).

The Dearing strain of serotype 3 reovirus is highly virulent after intracerebral inoculation (20). This pattern of neurovirulence after intracerebral inoculation has been shown to be primarily a property of the S1 gene product (the viral hemagglutinin) (20). When we inoculated this strain perorally into suckling mice, it was avirulent. Our study was undertaken to understand why the Dearing strain of serotype 3 reovirus was avirulent after peroral inoculation. We also were interested in defining how reovirus interacts with the mammalian host during passage through the gastrointestinal tract. We now report that: (*a*) there are marked differences between reovirus serotypes 1, strain Lang and 3, strain Dearing in their sensitivity to proteolytic enzymes and growth in intestinal tissue; (*b*) these differences are a property of the μ1C outer capsid polypeptide, which is encoded in genome segment M2; and (*c*) sensitivity or resistance of the μ1C polypeptide to proteolysis correlates with the ability to initiate systemic infection after administration of virus by peroral intubation. Thus the M2 dsRNA genome segment is a virulence gene.

Materials and Methods

Virus. Reoviruses serotype 1, strain Lang, and serotype 3, strain Dearing, have previously been described (22). Recombinant clones used in these experiments were prepared by infecting L cells with equal multiplicity of temperature-sensitive (ts) mutants of reovirus serotype 3, strain Dearing and clones derived from serotype 1, strain Lang (23, 24). The parental origins of each dsRNA segment, as well as the serotype 3 ts mutant used to generate each recombinant clone, are listed in Table I. All virus clones were double plaque purified and passaged on L cell monolayers twice. A third passage was grown in L cell spinner culture and purified according to published methods (22). The pure virus was collected from CsCl gradient and dialyzed against 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.4. The same purified viral stocks were used for in vitro and in vivo experiments.

Cells and Viral Titrations. Mouse L cells were maintained as previously described (22). All viral titrations were performed on L cell monolayers in Costar 6 well plates (3506; Costar Data Packaging, Cambridge, Mass.). For plaque assays, we used previously described methods (25) adapted for the smaller surface area. Titrations were performed at 37°C (CO_2 incubator 3331-2; National Appliance Co., Hollywood, Fla.).

Assays for Chymotrypsin. A stock solution of 1 mg/ml chymotrypsin (CHT) (C4129, three times crystallized; Sigma Chemical, Co., St. Louis, Mo.) was prepared. The specific activity of the CHT solution was determine by spectrophotometric assay using benzoyl-L-tyrosine-ethyl ester (BTEE) as substrate (26). The activity of aliquots of CHT stored at −70°C was determined at the beginning and end of each experiment. No change in activity was observed.

To measure CHT activity in intestinal tissue, the small intestine of 1- to 2-d-old mice was divided at the gastroduodenal junction and cecum and placed in 1 ml of distilled water. The samples were sonicated until a fine suspension was obtained. Because the BTEE specifically measures CHT activity, the intrinsic brush border enzymes on the surface of intestinal cells (27,

TABLE I

Genotype of Reovirus Clones

Serotype or recombinant	Serotype 3, strain Dearing ts mutant used as parent for recombinant	Parental origin of genome segments									
		Other capsid			Core					Nonstructural	
		M2	S1	S4	L1	L2	L3	M1	S2	M3	S3
1		1	1	1	1	1	1	1	1	1	1
3		3	3	3	3	3	3	3	3	3	3
65	tsC (447)	1	1	1	3	3	3	3	1	3	1
54	tsB (352)	1	1	3	3	1	1	1	3	3	3
63	tsB (352)	1	3	3	3	1	1	1	1	1	1
204	tsD (357)	1	3	1	3	1	1	1	1	1	1
402	tsE (320)	3	3	1	3	3	3	3	3	3	3
502	tsC (447)	3	3	3	1	3	3	3	3	3	3
602	tsC (447)	3	3	1	1	3	3	3	3	3	3
802	tsC (447)	3	1	3	1	3	3	3	3	3	3
94	tsD (357)	3	3	1	1	3	3	1	1	3	1
103	tsD (357)	3	1	1	1	3	3	3	3	3	3
140	tsG (453)	3	1	1	1	1	1	1	1	3	3

28) did not affect the assay. The spectrophotometric assay noted above was used to determine enzymatic activity. The protein content of intestinal samples were determined according to Lowry (29) using bovine serum albumin as a standard. The CHT activity was related to the protein content of small intestine/100 g body weight. To determine the amount of enzyme protein/U of CHT activity, the total small intestinal CHT activity was further correlated to the specific activity of purified CHT by modifying the method of Hummel (26). A standard curve of activity/μg of purified CHT was calculated and the intestinal activity was related to known concentrations of purified enzyme.

For in vitro CHT digestions, 250 μg of purified virus was diluted into 1.0 ml of buffer that contained 0.01 M NaCl and 0.01 M tris, pH 8.3. This virus solution was then further dialyzed against the same buffer, and 10 μl (2.5 μg of virus) was then diluted into 1 ml of a solution that contained the appropriate cation solution buffered with 0.10 M Tris, pH 8.3.

Reaction mixtures with 10 μg CHT were incubated for 1 h at 37°C. Controls were incubated in a similar manner without CHT. Each point represents the mean of two samples. At the end of the incubation period, samples were rapidly cooled to 4°C and 1% aprotinin (A6012; Sigma Chemical Co.) was added to terminate the digestion (this concentration of aprotinin inhibited the enzyme activity). Serial dilutions were made in gelatin saline and 0.10-ml aliquots were titered on L cell monolayers as indicated above.

Assays of Growth of Virus in Intestinal Tissue

INOCULATION OF VIRUS INTO STOMACH. BALB/c pregnant mice were obtained from The Jackson Laboratory, Bar Harbor, Maine and Laboratory Supply Co., Indianapolis, Ind. 1- to 2-d-old suckling mice were inoculated with suspensions of virus by passing a PE-10 polyethylene catheter (Intramedic polyethylene tubing inner diameter = 0.61 mm; Clay Adams, Div. of Becton, Dickinson & Co., Parsipanny, N. J.) through the mouth and into the stomach. To guarantee delivery of virus into the stomach, 0.06 ml of blue dye (Durkee Famous Foods, Cleveland, Ohio) was added to virus suspended in 10 ml gelatine saline. An inoculum of 0.05 ml that contained ~1×10^7 plaque-forming units (pfu) of the appropriate virus was delivered with a tuberculin syringe.

VIRAL TITERS IN INTESTINAL TISSUES AFTER PERORAL INOCULATION. Mice were killed on days 1, 2, and 3. Intestines were harvested from gastroduodenal junction to distal colon and washed three times in cold phosphate-buffered saline. The small intestine was then separated from the colon and placed in 0.50 ml gelatin saline at 4°C. Tissue suspensions were prepared for titration

by three sequential freeze-thaw cycles followed by sonic treatment for 20 sec to disrupt the remaining intact cells and viral aggregates. Virus was titered as above.

TITERS OF VIRUS AFTER INCUBATION IN VITRO WITH GASTRIC FLUIDS OR INTESTINAL TISSUE. 2-d-old mice were killed and stomach and intestine removed. The small intestine was separated from the stomach at the gastroduodenal junction and from the large intestine at the cecum. The stomach contents from two mice were pooled (~0.1 ml), diluted with an equal volume of solution that contained 0.03 M KCl and 0.01 M NaCl, and sonicated. The small intestines from two mice were pooled and sonicated until homogenized. Reovirus serotypes 1 and 3 (2.5 μg in 10 μl) were added to the gastric and small intestinal suspensions. An initial aliquot was taken for titration and samples were incubated at 37°C for 2 h. Aliquots of each sample were removed for titration at 1 and 2 h.

Mouse Survival. Multiple litters of newborn mice were inoculated by peroral intubation with each parental serotype and selected recombinant clones. Survival was determined by recording the number of animals alive up to 3 wk after inoculation. Statistical analysis using contingency chi-square was used to compare the recombinant clones that contained the serotype 3 S1 dsRNA segment with serotype 3, strain Dearing.

Results

Digestion by CHT Affects Reovirus Serotypes 1 and 3 Differently. The intestinal fluids contain several proteases including the acid proteases; i.e., pepsins (30) and the serine proteases trypsin and CHT (31). CHT is a major enzyme found in high concentration in the upper small intestine (31), and it is known to be capable of digesting the outer shell of mammalian reoviruses (11). To determine if CHT activity was present in newborn mice, we measured enzyme activity of sonicated 1- to 2-d-old mouse intestines. The CHT activity present in 1- to 2-d-old mouse intestine was found to be 41.6 ± 7 U/100 g body wt. This value is lower than the ~200 U/100 g body wt previously reported for newborn rats (28). To determine the amount of enzyme protein corresponding to 41.6 U, intestinal CHT activity was related to the specific activity of known concentrations of purified enzyme. The activity was equivalent to ~4 μg of purified CHT. This value is a minimum value because dietary proteins, natural substrates for CHT, were present in the in vitro reaction mixture. Thus, the pancreatic enzyme CHT is present in newborn mice. To determine the effect CHT has on the infectivity of reovirus serotype 1, strain Lang and serotype 3, strain Dearing, we examined CHT digestion in the presence of different concentrations of Na^+, K^+, and Cs^+. The concentrations of monovalent cations are known to affect the response of reovirus serotype 3, strain Abney (9–13). The cation concentrations were selected to represent: (*a*) in vitro conditions that yielded maximal proteolysis (Fig. 1; Table II A); (*b*) intracellular Na and K concentrations (Table II B); and (*c*) cation concentrations found in the upper gastrointestinal tract of newborn suckling mice (Table II C).

Maximum proteolytic digestion of the Abney strain of reovirus serotype 3 occurs in the presence of Cs^+ (12, 13). We have found that serotype 3, strain Dearing also undergoes loss of infectivity in the presence of Cs^+, whereas serotype 1, strain Lang is not significantly affected (Fig. 1, column a). Thus, the serotype 1, strain Lang is resistant to digestion by CHT, whereas the serotype 3, strain Dearing is sensitive to proteolytic digestion. When proteolytic digestion proceeds in salt concentrations that simulate the monovalent cationic content of intracellular fluids (Table II B) and that of the upper gastrointestinal tract (Table II C), similar but less marked losses of infectivity of serotype 3, strain Dearing occur.

Identifying the M2 dsRNA Segment as the Gene Responsible for the Differences in Response to

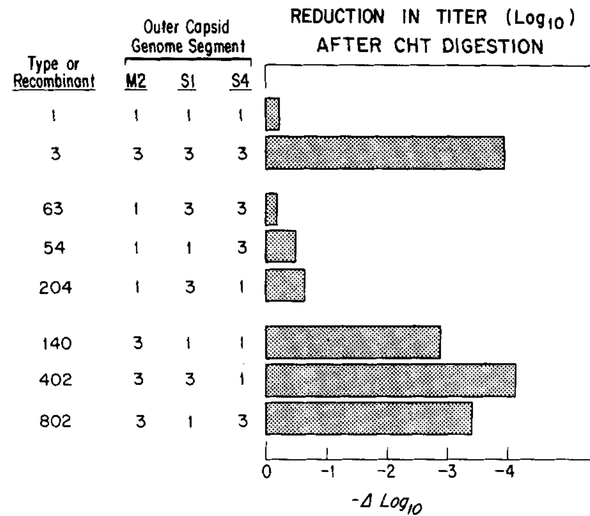

FIG. 1. Reduction in titer ($\log_{10}$) after CHT digestion. Selected viral stocks were incubated with CHT in the presence of Cs^+ as described in Table I. The parental origins of the genome segments encoding the three outer capsid polypeptides are listed.

Proteolysis. To identify the gene(s) responsible for the different responses of the reovirus serotypes to enzymatic digestion, we used several recombinant clones derived from crosses between reovirus serotypes 1, strain Lang and 3, strain Dearing and which consisted of different combinations of genomic segments derived from both parents ([23]; and Table I). The isolation of these recombinants and the methods used to determine the genotypes have been previously described (23, 24).

Clone 65 was studied first because all the dsRNA segments that encode outer capsid polypeptides (M2, S1, S4) were derived from serotype 1, whereas most of the dsRNA segments that encode internal and nonstructural polypeptides were derived from type 3 (Table I). The pattern of response to CHT digestion under all salt conditions was similar to the pattern seen with serotype 1, strain Lang, i.e., there was only a slight decrease in infectivity when digestion was carried out in the presence of CsCl and an increase in titer in conditions similar to gastric fluids (Table II). These results suggested that one or more components of the outer capsid were playing the primary role in sensitivity to CHT digestion.

Subsequent experiments focused on recombinants that segregated dsRNA segments encoding polypeptides of tne outer capsid (M2, S1, S4). The results with a number of recombinants excluded both the S1 and S4 segments and indicated a primary role for the product of the M2 dsRNA segment. In the presence of 0.10 M Cs^+, the salt condition that is optimal for the digestion of type 3, strain Dearing, the recombinants that contained an M2 dsRNA segment from serotype 1, strain Lang lost little infectivity; whereas the infectivity of recombinants with an M2 dsRNA derived from type 3, strain Dearing was reduced markedly (Fig. 1; Table II A). The role of the M2 gene segment in determining resistance to proteolytic digestion is best illustrated in recombinant 63. Recombinant 63 contains an M2 segment derived from serotype 1, and S1 and S4 segments (the other dsRNA segments encoding outer capsid proteins) from type 3; recombinant 63 behaves entirely like serotype 1 when exposed to

TABLE II

Effect of CHT Digestion in the Presence of Various Salts on the Infectivity of Reovirus Serotype 1, Strain Lang, Reovirus Serotype 3, Strain Dearing, and Reovirus Recombinants

Serotype or recombinant	Genes encoding outer capsid polypeptides			Salt conditions		
	M2	S1	S4	A. Maximal (0.10 M CsCl; 0.10 M NaCl)	B. Intracellular (0.145 M KCl; 0.010 M NaCl)	C. Upper gastrointestinal tract (0.030 M KCl; 0.010 M NaCl)
1	1*	1	1	−0.18	+0.34	+0.33
3	3	3	3	−3.92	−1.47	−1.35
65	1	1	1	−0.79	+0.01	+0.29
54	1	1	3	−0.45	+0.32	+0.43
63	1	3	3	−0.17	+0.36	+0.39
204	1	3	1	−0.59	+0.27	+0.36
402	3	3	1	−4.15	−1.75	−1.17
502	3	3	3	ND	−1.30	−1.40
602	3	3	1	ND	−1.80	−0.89
802	3	1	3	−3.39	−2.09	−1.04
94	3‡	3	1	−1.96	−1.15	+0.09
103	3‡	1	1	−3.04	−1.06	−0.16
140	3‡	1	1	−2.82	−1.30	+0.09

Reovirus serotypes 1, strain Lang, type 3, strain Dearing, and recombinant clones were incubated with CHT at 37°C for 1 h in one of three cation conditions. The conditions were selected to simulate Na^+ and K^+ content of selected body fluids: A, maximal conditions of digestion (Cs^+), B, intracellular, or C, upper gastrointestinal tract. After incubation, the samples were placed on ice and the CHT was inactivated with 1% aprotinin. The samples were titered on L cell monolayers as described in Materials and Methods. The numbers represent loss (−) or gain (+) in infectivity ($\log_{10}$) of samples treated with CHT compared with untreated controls. ND, not done.

* Numbers represent parental origin of outer capsid genome segments.

‡ M2 dsRNA segments that have a nontemperature-sensitive mutation.

proteolytic enzymes. Thus the only outer capsid protein derived from serotype 1, strain Lang in this enzyme-resistant recombinant is the μ1C polypeptide. In a similar way, recombinant 140 has an M2 segment derived from serotype 3 and every other structural gene derived from serotype 1; infectivity after proteolysis is reduced in a manner similar to that of the serotype 3 parent. In all other recombinants examined, the parental origin of the other two outer capsid polypeptides (coded for by the S1 and S4 genome segments) did not correlate with the pattern of reaction to CHT digestion. Similarly, all the core and nonstructural polypeptides were excluded. Thus the μ1C polypeptide is solely responsible for determining sensitivity or resistance to proteolysis in the presence of CsCl.

With salt conditions simulating those of an intracellular environment, the recombinants reacted to CHT digestion in a manner similar to but less marked than that found in the presence of CsCl (Table II b). Under these more-physiologic conditions, the μ1C polypeptide also determines resistance to proteolysis.

Because the overall goal of these studies was to evaluate viral entry through the gastrointestinal tract, CHT digestion was performed under conditions that closely simulated fluid contents of the stomach and upper small intestine. It has not been feasible to perform in vitro experiments directly utilizing the intestinal contents of

newborn mice because of the difficulty in obtaining sufficient amounts of intestinal fluid. Gastric contents, which consist largely of milk, are viscous and therefore dilution was required to achieve a liquid suspension. Gastric contents were collected and pooled from five suckling mice and were analyzed for concentrations of Na^+ and K^+ by standard flame photometric techniques (model 343; Instrumentation Laboratory, Inc., Lexington, Mass.). The concentrations of these two cations were: $Na^+ = 0.01$ M; $K^+ = 0.03$ M. Gastric contents were than diluted twofold (total vol: 200 μl) in 0.03 M K, 0.01 M Na, and 2.5 μg ($\sim 10^{7.5}$ pfu) of reovirus serotypes 1 and 3 were added. There was no change in titer of either reovirus serotypes 1 or 3 after 2 h of incubation at 37°C. When 2.5 μg of reovirus serotypes 1 and 3 were incubated at 37°C with sonicated whole small intestine (50 μl) that was obtained from 2-d-old mice, reovirus serotype 1 increased in titer ~0.7 $\log_{10}$, whereas serotype 3 decreased in titer ~1.2 $\log_{10}$ during the 2-h incubation period. Thus, incubation of reovirus serotype 3 with homogenates of small intestine, but not stomach, decreases the titer ~1.2 logs.

To determine if the pancreatic protease CHT might account for the decreased titer of serotype 3 in intestinal homogenates, we attempted to closely reproduce the physiologic salt conditions of the upper small intestine in vitro. Because of the small size of the intestine of newborn mice, we were unable to directly measure the concentration of cations present at the sites of entry of pancreatic secretions in the duodenum. It is known that the concentration of cations present in the upper small intestine depends upon the secretion of enzymes and sodium bicarbonate by the pancreas, the absorptive capacity of the intestinal epithelium, and the dietary intake of solids and fluid (32, 33). The capacity of the pancreas to develop a secretory phase is not mature in newborn rodents (34). Hence, dietary concentrations of cations, as measured in the stomach, assume a greater influence on the concentration of cations in the upper small intestine. The concentration of cations present in the stomach, as determined above, was used as an approximation of the cation content of the upper duodenum. When virus was treated with CHT in the presence of these concentrations of cations, the titer of serotype 3, strain Dearing was reduced. We detected a slight increase in titer in serotype 1 (Table II C). Although all recombinants that contained an M2 segment derived from serotype 1 were similar to serotype 1, certain recombinants that contained an M2 dsRNA segment derived from serotype 3 more closely resembled serotype 1 than serotype 3 (Table III, recombinants 94, 103, and 140). The aberrant behavior of these three recombinants was initially unexplained. However, in attempting to understand these results, three prior observations were noted: (*a*) These three recombinants were all derived from crosses involving tsD(357) and tsG(453) (Table I; and [18, 24]); (*b*) both tsD and tsG mutants had previously been shown to synthesize μ1C polypeptides of abnormal mobility on polyacrylamide gel electrophoresis (35) and, in fact, the μ1C polypeptide of tsD(357) produces altered tryptic digestion products (36); and (*c*) in both cases, the aberrant μ1C polypeptide is not the site of the ts mutation (tsD = L1, tsG = S4 [23, 24]) but, rather, is the location of a silent nontemperature-sensitive mutation (35). For these reasons, we hypothesized that the mutation causing the abnormal μ1C polypeptide in tsD357 and tsG453 might have conferred resistance to CHT digestion. This has been shown to be the case (D. H. Rubin. Unpublished data.). Thus the M2 dsRNA segment in recombinants 94, 103, and 140, although derived from serotype 3, was mutated in tsD(357) and tsG(453) and thus behaves differently from the M2 dsRNA segment derived from the wild-

type serotype 3, strain Dearing parent. This fact is noted in Table II by recording the serotype 3 M2 segments derived from mutants with altered μ1C polypeptides with double daggers (‡). Thus, the altered response of recombinants 94, 103, and 140 to CHT digestion in cation conditions that simulate gastric content is a result of a previously unrecognized property of the nontemperature-sensitive mutations in the M2 dsRNA segments derived from tsD(357) and tsG(453). It should also be noted that the response to CHT digestion of these three recombinants in the presence of Cs^+ or high K^+, although similar to the wild-type serotype 3 μ1C, is less marked.

The M2 dsRNA Segment Determines the Pattern of Viral Growth in Intestinal Tissues after Gastric Inoculation of Reovirus Serotypes. To determine whether the difference in sensitivity of reovirus serotypes 1 and 3 to proteolytic enzymes plays an important role in the entry of viruses through the gastrointestinal tract, we studied inoculation of virus into 1- and 2-d-old BALB/c mice. Because of the small size of these mice, it was difficult to be certain that virus was introduced solely into the stomach. Because inoculation of reovirus serotype 3 into the peritoneal cavity often results in a lethal infection (D. Rubin. Unpublished observations.), we had to insure that virus was properly administered. To insure delivery of virus solely to the stomach, virus was diluted with gelatin saline that contained a nonadsorbable blue dye. Virus was inoculated via a polyethylene catheter into the stomach. We were then able to determine, by examining the nearly transparent abdominal wall for the location of the blue dye, if performation of the intestine, aspiration into the lungs, or other mishaps had occurred (Fig. 2).

When either low (10^3 pfu/mouse) or high (10^7 pfu/mouse) viral inocula were used, titers of virus recovered from intestinal tissues 6-h–8-d after infection were higher with serotype 1 than with serotype 3. After inoculation of serotype 1 infection at a dose of 10^3 pfu/mouse, titers of $\sim 1 \times 10^6$ pfu/ml serotype 1 virus could be recovered at 48 h. No infectious virus could be recovered after inoculation with serotype 3 at this dose. After inoculation of virus at doses of 10^7 pfu/mouse, virus could be recovered from intestinal tissue for both serotypes 1 and 3. This dose corresponded to the virus concentration used in the in vitro studies. The higher dose was selected for detailed analysis in vivo.

Because we were mainly concerned with entry of virus through the gastrointestinal tract, our detailed studies were done on days 1–3 after infection. Serotype 1 virus significantly increased in titer during the first day and reached a titer of $\geq 10^8$ pfu/ml at 24 h. Serotype 3 declined to a titer of $\sim 10^6$ pfu/ml at 24 h (Fig. 3a). Thus the serotype 1, strain Lang virus, which is resistant to CHT digestion in vitro, grows more efficiently in intestinal tissues.

To identify the genetic basis of the patterns of viral growth, we studied the recombinant clones previously analyzed in vitro. Clones 54, 63, 65, and 204 all contain an M2 dsRNA of serotype 1 and resemble serotype 1 in their growth properties (Fig. 3b). In a similar fashion, recombinants 402, 502, 602, and 802 all contain an M2 dsRNA of serotype 3 and resemble serotype 3 in their loss of titer (Fig. 3C).

Recombinants containing the altered (CHT resistant) serotype 3 μ1C polypeptide (Table II, recombinants 94, 103, and 140) displayed in vivo an early increase in titer, like serotype 1 (Fig. 3d). However, at a later time, there was more variability in their pattern of growth (for example see the titer of clone 94 at day 3). Thus the altered in vitro behavior of the μ1C polypeptide to proteolytic digestion corresponds to the

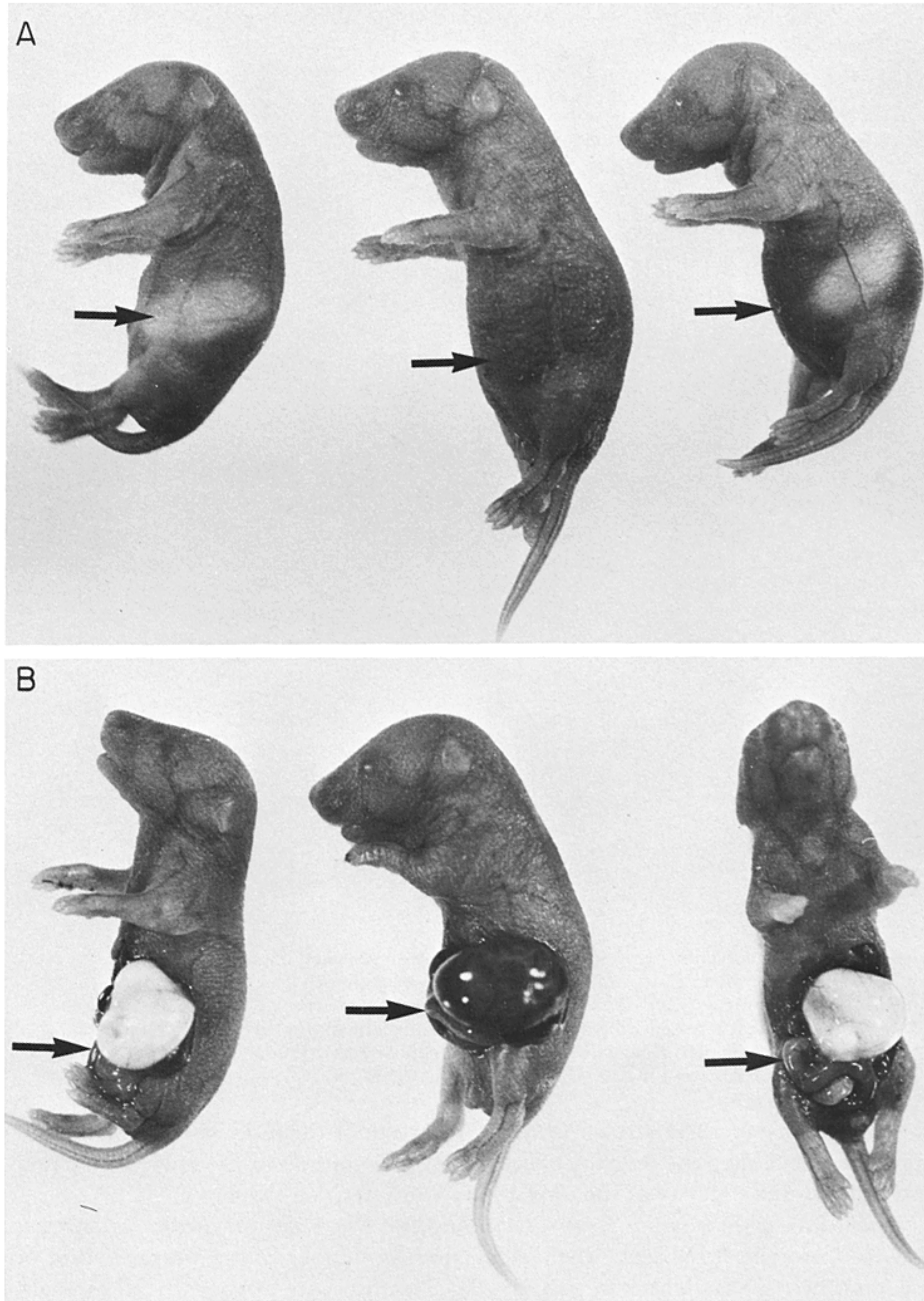

Fig. 2. External and internal appearance of mice after administration of virus by peroral intubation. (A) External appearance of mice: left—control mouse showing milk prominently visible in stomach; center—mouse inoculated successfully as indicated by darkening of stomach as seen through abdominal wall; and right—mouse accidentally inoculated into peritoneal cavity showing darkening of peritoneal cavity outlining the whitish milk in stomach. Arrow points to whitish milk in stomach (left); stomach darkened by correctly inoculated virus suspension containing blue dye (center); and darkening of peritonium by improperly inoculated stock (right). (B) Appearance of abdominal viscera of mice shown in (A) after abdominal incision. Arrow points to normal peritoneum and small intestine (left); normal stomach and small intestine darkened with blue dye in lumen (center); and staining of peritonium because of perforation from improper inoculation (right). × 4.

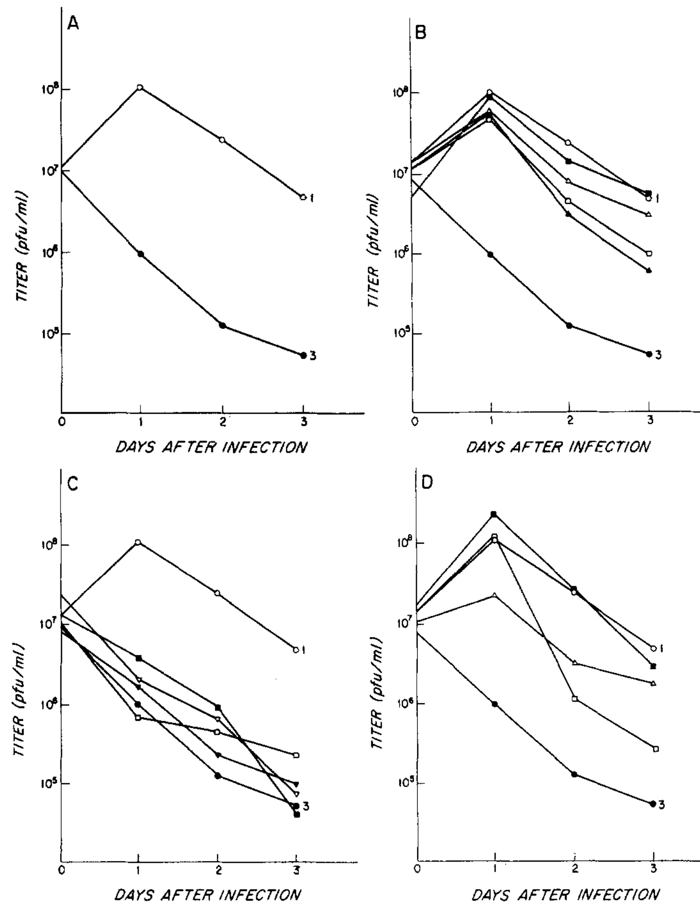

FIG. 3. Multiplication of reovirus serotype 1, reovirus serotype 3, and selected recombinant clones in intestinal tissue after administration of virus (~10^7 pfu/mouse) by peroral intubation. The method of inoculating mice and titering of virus from intestinal tissue is indicated in Materials and Methods. Each point represents the mean of three to nine animals. (A) Reovirus serotype 1 (○); reovirus serotype 3 (●); (B) recombinants 54 (■), 63 (□), 204 (▲), 65 (△); (C) recombinants 602 (■), 502 (□), 802 (▲), and 402 (△); (D) recombinants 140 (■), 94 (□), and 103 (△).

enhanced ability of these strains to grow in intestinal tissue in vivo. This finding strongly suggests that the resistance of the μ1C polypeptide to proteolytic digestion contributes to the capacity of the virus to grow in intestinal tissue.

The Capacity of Reovirus to Grow in the Intestine Determines the Pattern of Systemic Infection. Serotype 3 reovirus, when inoculated by various routes (intracerebral or subcutaneous, e.g.) is highly virulent (37, 38). Death occurs in virtually all animals, even after inoculation of low doses, because virus infects neurons and produces a necrotizing encephalitis (20). Infection with serotype 1 reoviruses is relatively benign because the virus infects ependymal cells and produces an acute ependymitis (39). This difference in pattern of illness is a function of the σ1 polypeptide—the viral hemagglutinin—encoded by the S1 dsRNA segment (20). To determine whether the differences in capacity of reovirus serotypes 1 and 3 to grow in intestinal tissue affects viral virulence, we analyzed the effect of administering serotype 1, strain Lang and

serotype 3, strain Dearing by peroral intubation on their capacity to produce lethal central nervous system disease. Because serotype 1 reovirus does not produce lethal infection, even after direct intracerebral inoculation, it was not surprising that very few animals died when inoculated perorally (Table III). However, even after inoculation by peroral intubation of high doses of serotype 3, strain Dearing, very few animals died (Table III) despite the virulence of serotype 3 when inoculated intracerebrally or subcutaneously (20, 37).

The role of the product of the M2 dsRNA segment in determining the outcome of inoculation of reovirus into the stomach was dramatically illustrated by experiments with recombinants such as 63 and 204 that contained an M2 segment from serotype 1 (enzyme resistant) and an S1 segment from serotype 3 (neurotropic). In contrast to either parental serotype, recombinants 63 and 204 were virulent (Table III). Recombinants that contain serotype 3 M2 and S1 dsRNA segments, such as 402, 502, and 602, generally behaved in a fashion similar to the serotype 3 parent and produced a nonlethal infection (Table III). Recombinant 94 contained an S1 dsRNA from serotype 3 and the mutant M2 dsRNA from ts D357 (23). It behaved more like the serotype 1 parent when digested in vitro in monovalent cations that simulated gastric contents (Table II, column C) and when analyzed for growth in intestinal tissue (Fig. 3 d). Recombinant 94 was significantly more virulent than serotype 3, strain Dearing, yet not as virulent as recombinant clones containing a serotype 3 S1 dsRNA and a serotype 1 M2 dsRNA. Thus the altered serotype 3 M2 dsRNA segment of clone 94

TABLE III

Survival of Suckling Mice after Inoculation by Peroral Intubation with Reovirus Serotype 1, Strain Lang, Reovirus Serotype 3, Strain Dearing and Recombinants

Serotype or recombinant	Genotype		No. mice dead/total	P value*
	M2	S1		
			%	
1	1	1	1/33 (3)	
3	3	3	1/42 (2)	
54	1	1	0/11 (0)	
63	1	3	25/72 (35)	<0.0002
204	1	3	27/49 (55)	<0.0001
94	3	3	10/48 (21)	<0.02
103	3	1	0/21 (0)	
402	3	3	2/24 (8)	>0.65
502	3	3	1/23 (4)	>0.70
602	3	3	4/33 (12)	>0.20

Mice were examined for 3 wk after administration of 10^7 pfu of virus/mouse by peroral intubation. Most deaths occurred between days 9 and 15. Histologic examination of brains collected from sick animals that were infected with recombinant clones that contained an S1 segment derived from serotype 3, revealed neuronal destruction similar to that previously reported (20).

* The survival of the mice inoculated with recombinant clones that contained the serotype 3 S1 gene were compared with the survival of mice inoculated with serotype 3 as indicated in Materials and Methods. *P* values <0.05 indicate significant differences between the recombinant and serotype 3.

has increased the capacity of the Dearing strain to grow and ultimately produce fatal disease.

The titers of virus recovered in the brain after viral inoculation by peroral intubation corresponded to those expected from the patterns of virulence noted above; the highest titers were found after inoculation with recombinants that contained M2 of serotype 1 and S1 from serotype 3; whereas lowest titers were found after inoculation of viruses that contained an M2 of serotype 3 and an S1 of serotype 1 (D. H. Rubin. Unpublished data.).

These experiments directly demonstrate that combining a CHT-resistant M2 genome segment from serotype 1 (or a CHT-resistant mutant M2 genome segment derived from a mutant of serotype 3) with the neurovirulent S1 genome segment from serotype 3, results in a virus that is virulent after administration of virus into the stomach. These recombinants are considerably more virulent than either of the parental serotypes.

Discussion

The results of this study indicate that the M2 dsRNA genome segment, which encodes the major μ1C outer capsid polypeptide, is responsible for the difference in virulence when the Dearing strain is inoculated perorally as compared to the intracerebral route. After inoculation of virus directly into the upper intestinal tract, the Dearing strain behaves as an avirulent strain, whereas inoculation by the intracerebral route results in a highly lethal (virulent) infection. The differences in response have been studied in vitro and in vivo. With recombinant clones derived from crosses between reovirus serotype 1, strain Lang and serotype 3, strain Dearing, we have been able to show that the M2 dsRNA segment, which encodes the μ1C polypeptide, is the segment responsible for differences in response to CHT in vitro. The serotype 1, strain Lang is resistant to inactivation either by intestinal homogenates or by the serine protease CHT in cation conditions that simulate the upper intestines; serotype 3, strain Dearing is highly susceptible to inactivation both by intestinal homogenates and by treatment with enzymes in vitro. Recombinants that contain the M2 derived from the serotype 1, strain Lang parent behaved like serotype 3. The exceptions to this behavior involved recombinants in which the M2 segment was derived from serotype 3 clones that contained mutations in the M2 segment that had conferred increased resistance to CHT digestion.

That in vitro digestion of reovirus by CHT mimics the growth pattern of reovirus serotypes and recombinants in the intestine strongly suggests that CHT is playing a role in the pattern of viral growth in vivo.

The in vitro responses to digestion by CHT paralleled the pattern of growth of virus in the small intestine as well as the ability of the virus to enter the systemic circulation and ultimately cause fatal encephalitis. After peroral inoculation, the serotype 1, strain Lang grew in intestinal tissue, whereas the titers of serotype 3, strain Dearing progressively declined. Furthermore, in the case of the serotype 3 strain that is highly neurovirulent after parenteral inoculation, death does not occur when virus is introduced into the intestinal tract. Recombinants that contain a protease-resistant M2 gene product derived from serotype 1 reovirus can resist proteolysis of enteric fluids and, if they contain a serotype 3 hemagglutinin (S1 gene product), are virulent after inoculation of virus perorally. The M2 genome segment is thus a virulence gene

because it directly affects the ability of the reoviruses to cause disease after introduction into the gastrointestinal tract.

How could sensitivity to CHT affect the intestinal phase of infection? It seems to us that there are two possibilities. First, proteases may affect viruses directly as they pass through gastrointestinal fluids. Secondly, they may affect the absolute titer of virus after release from sites of primary multiplication within the intestinal tract. Clearly, both mechanisms may be taking place. We have shown that direct inactivation of virus by intestinal homogenates causes a reduction of viral titers. However, the persistence of serotype 3 reovirus in intestinal tissues for 3 d, albeit in reduced amounts, strongly suggests that serotype 3 virus is being synthesized. Because the precise site in the intestinal tract of primary viral multiplication has not been defined, we have not yet resolved this issue. It is quite likely, however, that by whatever mechanism, proteases are participating in maintaining viral levels below a critical threshold that is required for effective spread to the systemic circulation and ultimately to the brain.

The precise site of proteolysis of virus in vivo, whether in the intestinal lumen or at the surface of intestinal cells (27, 31), is also not known. Gastrointestinal proteolytic enzymes are produced primarily in the stomach and the pancreas. Pancreatic enzymes are secreted into the small intestine where they play a major role in digestion of dietary proteins (31, 40). Proteolytic enzymes are found not only in intestinal secretions but are also major components of blood serum (complement, clotting factors), major products of cells that are important in host defense (macrophages), and are distributed in most organs of the body (41, 42).

These experiments indicate how gastrointestinal disease and nutrition could affect viral entry. Diseases with reduced secretion or activity of gastric or pancreatic enzymes such as cystic fibrosis, kwashiorkor, chronic pancreatitis, postgastrectomy syndrome, could, by reducing the amount of proteolytic enzymes available (43–45), increase susceptibility of the host to enteric viruses. Furthermore, dietary factors that may alter cation concentration or the activity of proteolytic enzymes, (i.e. protease inhibitors), could possibly modify the entry of otherwise nonvirulent viruses.

These results thus define a second virulence gene for the mammalian reoviruses and demonstrate how specific viral components interact with the environment of the intestinal tract. Further exploration of viral host interactions may have important implications for understanding the relationships of diet, gastrointestinal disease, and viral pathogenesis.

Summary

The mammalian reoviruses (serotype 1, strain Lang and serotype 3, strain Dearing) differ in their sensitivity to digestion by chymotrypsin. We have found that the M2 double-stranded RNA (dsRNA) genome segment (encoding the μ1C outer capsid polypeptide) is responsible for this property. In addition to determining response to protease treatment in vitro, we have found that the M2 genome segment also determines the ability of these two viruses successfully to initiate local and systemic infection in newborn mice after peroral inoculation. Thus the M2 dsRNA segment defines a new virulence gene of the mammalian reoviruses.

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