Studies on attenuation of rotavirus

A comparison in piglets between virulent virus and its attenuated derivative

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Summary. The development of rotavirus vaccines against acute gastroenteritis for human infants has been accorded a very high priority. Several vaccine candidates all of which are live cultivated strains of animal origin have been tested in humans. However the nature of attenuation of these viruses for humans is unknown. In this study we have attenuated a pig rotavirus by 15 sequential passages in cell culture after which the virus no longer causes diarrhoea in piglets. The pathogenesis of infection of the attenuated rotavirus strain (AT/ 76 P15) in gnotobiotic piglets was compared with that of the virulent parent strain (AT/76). The pattern of virus replication in the small intestine was judged by histology, disaccharidase assay, immunoperoxidase labelling of gut sections using group A specific rotavirus antibody, and rotavirus antigen assay of gut contents. The parent strain caused variable but extensive infection that resulted in the complete destruction of mature small intestinal enterocytes and villous contraction within 3 days. Membrane bound digestive enzymes were lost, and profound watery diarrhoea and dehydration resulted in causing piglets to become moribund. In contrast attenuated virus appeared to propagate at a much slower pace. Fewer infected epithelial cells were detected at any one time. Destruction of enterocytes was never extensive enough to cause marked mucosal changes in histology. Membrane bound digestive enzymes remained near normal levels and there was little or no diarrhoea. Virus replication ceased after 6 days.

It is concluded that attenuation of the porcine rotavirus strain studied was associated with its decreased ability to propagate in enterocytes after adaption to culture.

Introduction

Group A rotaviruses vary in their pathogenicity for different species of mammals and birds, causing a clinically acute gastroenteritis in human infants and in the

young of most mammalian species, including gnotobiotic piglets [1]. Rotaviruses are considered host specific, with several serotypes existing for each host [1, 2]. Variations exist in terms of prevalence and virulence among serotypes [3, 4] which may provide a spectrum of severity of disease.

The development of rotavirus vaccines has been assigned a very high priority [5], and attempts so far have focused on the deployment of avirulent live oral vaccine strains. The ideal vaccine would be one eliciting a broad spectrum serotypic immune response [6]. Avirulent or attenuated rotaviruses are readily obtained either by using strains of different animal origin [7], or after sequential propagation in cell culture [8], or both [9]. A limited degree of cross-species infection among group A rotaviruses can be achieved, usually under laboratory conditions, causing asymptomatic or transient infections [10–12]. Rotaviruses also appear to attenuate rapidly upon in vitro cultivation. Another potential source of vaccine strains for use in humans are the so called "nursery strains" [13–15], which may be naturally attenuated. As with many other viruses, the nature and mechanism of attenuation of rotavirus is unknown. Clearly changes occur during adaption to in vitro propagation in cell culture, that render the virus less pathogenic to the host. There are several levels at which to investigate rotavirus attenuation. At the cellular level, changes in the number of infected cells, their distribution in the small intestine and their survival could be compared in the homologous host using wild type and cultivated strains. At the subcellular level, reduced affinity to cell receptors, reduced or defective (interfering?) production or assembly of particles could be sought. At the molecular level, changes in RNA and polypeptide sequences could be examined, particularly of VP4 and VP7.

In order to gain some insight into the mechanism of attenuation in terms of mucosal alteration and clinical outcome, we have attenuated a virulent pig rotavirus by sequential propagation in cell culture and compared piglets infected with the attenuated virus with piglets infected with the parent strain. This study included a qualitative and quantitative comparison of clinical and mucosal parameters measured over 3 days after inoculation of gnotobiotic piglets infected with either strain.

Materials and methods

Viruses

Rotavirus strain AT/76 was originally identified in faeces from a piglet involved in an outbreak of diarrhoea in farm animals in Victoria, Australia [16], and was passaged 8 times in gnotobiotic piglets. This strain was shown by electron microscopy and gel electrophoresis to be free of other viruses [17].

Strain AT/76 from 10% (w/v) PBS (pH 7.2) suspension of filtered gut contents of an infected piglet was used as inoculum for adaptation of the porcine strain to cell culture. Virus was initially adapted to growth in MA-104 cells in roller tubes, after pre-treatment of virus inoculum with trypsin (5 μg/ml) [18] was subsequently adapted to stationary culture, and was passaged 15 times in MA-104 cells in medium containing trypsin, using a multiplicity of infection of 0.1 each passage. The cell culture supernatant of the 15th passage was

designated AT/76P15. This was purified by firstly mixing cell culture supernatant with one-fifth volume of hydrofluorocarbon (Arklone, ICI Laboratories) followed by homogenization in a Waring blender and centrifugation at 10,000 g for 10 min at 4 °C. The aqueous phase was collected and ultracentrifuged at 100,000 g followed by resuspension of the pellet in 0.01 M Tris-HCl pH 7.4 with 10 mM $CaCl_2$ and 15 mM NaCl. The numbers of infectious particles per ml of AT/76 and AT/76P15 were assessed using immunofluorescence to detect MA-104 cells infected with rotavirus.

Sixteen gnotobiotic piglets were inoculated orally with one ml of AT/76P15 preparation, adjusted to contain approximately 10^3 fluorescence forming units (FFU). Piglets inoculated with strain AT/76 initially received one ml of filtered gut contents adjusted to contain approximately 10^3 FFU. This was subsequently diluted tenfold to contain approximately 10^2 FFU when the earlier dose proved lethal.

Animals

The derivation and maintenance of gnotobiotic piglets have been described previously [19]. Briefly, 27 newborn piglets derived from 3 litters were used in this study. They were inoculated with one of 2 strains of rotavirus (Table 1) within 24 h after delivery, when it was clear that they were healthy and drinking well. The piglets were observed 3–4 times per day for signs of anorexia, vomiting, diarrhoea and other signs of illness. They were euthanized by intravenous injection of sodium pentobarbitone one to 6 days after inoculation.

Necropsy procedure

The intestinal tracts of piglets were examined for gross pathological changes. Sections were taken from 11 equally spaced sites along the small intestine and from 2 sites in the large intestine. One part of each section was immediately fixed in buffered formalin, and later sectioned for light and electron microscopy, and for peroxidase-antiperoxidase (PAP) immune staining [20]. Mucosa was scraped from the second part of each of the 11 small intestinal sections, and was used to quantitate the amount per site of rotavirus antigen, and the activity of membrane bound lactase.

Determination of rotavirus concentration in gut tissue

Gut scrapings were prepared as 20% (w/v) suspensions in phosphate buffered saline (PBS), homogenized, subjected to one freeze-thaw cycle, then centrifuged at 10,000 g for 2 min. The supernatant was tested in an enzyme-immunoassay (EIA) using a group A rotavirus specific monoclonal antibody [21]. Briefly, 25 µl of gut homogenate supernatant was added in duplicate to 75 µl PBS-tween 20 containing 2.5% skim milk powder (PBST-SMP) in wells coated with rabbit anti simian rotavirus SA 11 antisera, and singly to wells coated with preimmune rabbit sera. After an overnight incubation at 4 °C, wells were washed and Group A specific monoclonal antibody diluted in PBST-SMP was added to all wells. After incubation at 37 °C for 150 min, wells were washed and rabbit antimouse-horseradish peroxidase conjugate (Silenus Laboratories, Australia) diluted in PBST-SMP was added. After incubation at 37 °C for 90 min, wells were washed and a substrate containing 3,3',5,5' tetramethyl benzidine (Sigma) was added. Optical density of reactions were determined using a Titertek spectrophotometer set at 450 nm.

In order to determine the concentration of rotavirus in each gut sample, a standard curve was constructed using OD measurements obtained with doubling dilutions of AT/76P15 of known FFU/ml, i.e., the optical density assessed at OD450 was plotted against the FFU/ml of each doubling dilution. After determination of the OD450 measurements of unknown samples, the FFU equivalent could be calculated by reference to the standard curve and expressed as FFU equivalent/ml/g wet weight.

Measurement of lactase activity

Gut scrapings from each of the small intestinal sites were processed for measurement of membrane-bound lactase activity expressed as units per g wet weight using the method of Dahlquist [22].

Results

Clinical outcome

Nine piglets were inoculated with 10^2–10^3 FFU strain AT/76 (Table 1). Two piglets were killed within 24 h of infection and before diarrhoea was apparent. The other seven piglets developed signs of anorexia, vomiting and diarrhoea

Table 1. Summary of experimental inoculation of piglets with virulent (AT/76) and attenuated (AT/76P15) pig rotavirus

RV strain	No. of pigs	Killed days p.i.	Diarrhoea[a]	Mucosal changes[b]	Viral antigen[c]	Lactase activity[d]
AT/76	2	1	—	1+	6.5×10^7 $(3.6$–$11.1 \times 10^7)$	31.0 $(19.2$–$47)$
	3	2	2+	2+	3.8×10^7 $(3.3$–$4.5 \times 10^7)$	1.3 $(0.5$–$1.5)$
	3	3	4+	4+	10.3×10^7 $(7.1$–$12.8 \times 10^7)$	1.2 $(0.5$–$2.5)$
	1	4	4+	4+	ND	ND
P15	2	1	—	—	$< 10^4$	22.2 $(14$–$33.3)$
	2	2	—	—	3.0×10^4 $(2 \times 10^4$–$4 \times 10^5)$	18.4 $(5.1$–$33.2)$
	4	3	—	—	2.9×10^7 $(3 \times 10^7$–$7 \times 10^7)$	14.5 $(3.8$–$36.4)$
	2	4	1+[e]	+	ND	ND
	2	5	1+[e]	+	ND	ND
	2	6	—	—	ND	ND
C	2	2	—	—	$< 10^4$	17.8 $(12.7$–$32)$
	2	3	—	—	$< 10^4$	15.2 $(11.3$–$29)$

[a] *1+* Mild diarrhoea; *2+* watery diarrhoea with vomiting; *3+* watery diarrhoea with dehydration; *4+* as above with severe anorexia

[b] + Swollen villous tips and vacuolated cells; *1+* as above with focal shortening of villi; *2+* as above with focal villous atrophy and fusion, and loss of mature enterocytes; *3+* as above over 1/2 of small intestine; *4+* as above 2/3 of small intestine

[c] Expressed as mean (range in brackets) FFU equivalent/g wet weight EIA of all sites of all pigs

[d] Expressed as mean (range in brackets) as IU/min/g of wet weight of 11 sites of all pigs

[e] One of 2 piglets had mild diarrhoea

ND Not done

C Control

within 46 h of inoculation. Despite the low infectious dose, affected piglets deteriorated progressively during three days post-infection, with only one piglet surviving up to 4 days. Fourteen piglets were fed 10^3 FFU of the cell culture attenuated strain AT/76P15. Only two (from the same litter) developed mild diarrhoea 4 and 5 days after inoculation, respectively (Table 1). Twelve of the piglets showed no clinical signs of infection during 1–6 days of observation.

Necropsy findings

Table 1 provides a comparison in terms of clinical outcome and mucosal changes in piglets infected with either AT/76 or its attenuated derivative AT/76P15.

In AT/76 infected piglets killed within 24 h of infection the small and large intestines were filled with whitish fluid despite lack of diarrhoea at this stage. Mucosal changes were minimal along the entire length of the small intestine in these animals although the presence of viral antigen, as observed by PAP (Table 2) and measured by EIA (Table 1), was extensive. Most of the histological changes were confined to the tips of the villi which appeared swollen. The enterocytes were vacuolated and contained large amounts of viral antigen. Lactase activity appeared normal.

Two days after inoculation, following onset of diarrhoea, mucosal changes were moderate with focal villous atrophy and villous fusion, and some loss of mature cells. Changes were largely restricted to the jejunum and upper ileum. Cells containing viral antigen were present throughout the small intestine although in variable numbers in different sites and in different piglets (Table 2). Lactase activity was severely reduced.

Three days after inoculation the mucosa was severely affected along most of the small intestine with profound villous atrophy and fusion. The lamina propria contained dense collections of inflammatory cells and was coated with irregular, vacuolated immature enterocytes. Lactase activity was markedly reduced. Numbers of cells containing viral antigen were markedly reduced.

Only one piglet remained well enough to survive 4 days. At necropsy this piglet had total villous atrophy. Very little viral antigen was seen in single cells (Table 2).

Piglets infected with strain AT/76P15 showed no macroscopic evidence of eneteritis at necropsy regardless of duration of infection. Only minimal histological changes were detected at any time and included varying degrees of vacuolation and loss of cells. Occasional oedema at the tips of villi was seen in the small intestines of piglets sampled 4–5 days post infection. Immunoperoxidase results showed a few virus containing cells scattered in small groups in the proximal small intestine after 24 h. Piglets sampled from day 2–5 showed gradual spread of infection in cells throughout the small intestine. A few virus containing cells were seen in mid jejunum on the 6th day. Rotavirus antigen was detected by EIA in piglets killed 48–72 h post infection. Activity of membrane bound lactase remained within normal range in sections of the intestine 24–72 h post infection.

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Table 2. Qualitative distribution of viral antigen, as detected by Peroxidase-Antiperoxidase (PAP), and severity of histological changes (Hs) observed in 11 small intestinal sites of piglets inoculated with pig rotavirus strain AT/76 (9 pigs) or its attenuated derivative AT/76P15 (14)

Strain	No. of pigs	Days p.i.		Sites from duodenum (1) to terminal ileum (11)										
				1	2	3	4	5	6	7	8	9	10	11
AT/76														
	2	1	PAP[a]	4+	4+	3+	3+	3+	3+	3+	2+	2+	1+	1+
			Hs[b]	1+	1+	1+	1+	1+	+	+	1+	1+	2+	2+
	2	1	PAP	3+	2+	3+	4+	3+	3+	2+	1+	2+	2+	2+
	1	2	PAP	+	+	1+	1+	2+	2+	2+	1+	1+	1+	1+
	1	3	PAP	2+	2+	2+	3+	2+	1+	1+	1+	1+	+	+
	2	3	PAP	+	1+	1+	1+	2+	1+	1+	1+	1+	+	+
			Hs	1+	1+	4+	4+	4+	4+	4+	4+	2+	1+	−
	1	4	PAP	1+	1+	+	+	+	+	+	+	+	+	−
			Hs	2+	3+	4+	4+	3+	3+	2+	3+	4+	3+	2+
AT/76P15														
	2	1	PAP	1+	2+	+	+	−	−	−	−	−	−	−
	1	2	PAP	1+	1+	1+	1+	1+	−	+	+	−	−	−
	1	2	PAP	1+	1+	1+	1+	2+	3+	4+	3+	2+	2+	2+
	3	3	PAP	1+	1+	1+	+	+	+	+	+	+	+	+
	1	3	PAP	1+	2+	2+	2+	2+	1+	1+	1+	1+	2+	2+
	4	4 & 5	PAP	1+	2+	1+	1+	1+	1+	1+	1+	1+	1+	1+
			Hs[c]	−	−	−	+	+	−	−	−	−	−	−
	2	6	PAP	+	+	1+	+	+	+	−	−	−	−	−

[a] PAP: + 1–2% of mature enterocytes contain viral antigen; *1+* 5–20%; *2+* 20–40%; *3+* 40–60%; *4+* 60–80%; − none detected

[b] Hs: + Swollen villous tips and vacuolated cells; *1+* as above with focal shortening of villi; *2+* moderate but focal villous atrophy and fusion and some loss of mature cells; *3+* as above with more severe changes; *4+* as above with the mucosa flattened and cellular; − no significant lesions observed

[c] Significant mucosal changes were only observed in 2 of these animals

Table 1 summarizes the correlation between diarrhoea, degree of mucosal damage, and amount of viral antigen measured for the first 3 days after inoculation in animals infected with either AT/76 or its attenuated derivative. It also shows an inverse relationship between these parameters and levels of membrane bound lactase activity measured in piglets infected with strain AT/76.

Table 2 lists in detail the distribution of viral antigen (detected by PAP) along the 11 segments of small intestine samples in relation to histological

changes observed for 1–6 days following inoculation with AT/76 or AT/76P15. Piglets infected with AT/76 were infected along the entire length of gut with maximal infection in the proximal half of the small intestine during the first 48 h post infection. Numbers of detectable cells infected decreased during the latter 48 h. Histological damage was minimal during the first 48 h and largely detected in mid small intestine. Maximal histological change was detected 72–96 h post infection. Piglets infected with AT/76P15 showed rotavirus antigen distributed along the entire length of the small intestine in most piglets 48–96 h post infection usually affecting only 5–20% of epithelial cells. Small numbers of cells remained infected even six days post infection in the proximal small intestine. Histological changes were only observed in piglets in two sections of mid small intestine 4–5 days after inoculation.

Discussion

This study clearly illustrates that the attenuated pig rotavirus failed to cause disease because fewer epithelial cells were infected at any one time compared with the parent strain. The "growth curve" of the parent strain was completed in three days. By this time the entire surface of mature small intestinal epithelium had been destroyed by virus and was being replaced, mostly with new immature cuboidal cells. Presumably, in order to facilitate the process of coating, villi had contracted and cross-bridging and fusion between them resulted from rapid migration from the crypt of poorly differentiated, still dividing, cells. The effect this had on the physiology of digestion was apparent; after a lag period of 24 h, the membrane bound digestive enzymes were lost, and the normal absorptive cells were replaced by less mature cells. The overall absorptive surface as a result of villous contraction, had markedly diminished. With such rapid cellular destruction, the mucosa was unable to recover sufficiently to maintain minimal capacity for disaccharidase digestion and absorptive needed for survival. Although some absorption must have taken place in the unaffected large bowel, it was not sufficient to compensate for the functional loss of the small intestine. It is also likely that in the interim, between rapid cellular loss and its replacement, leakage of plasma proteins and electrolytes from the mucosa may have further contributed to movement of fluids from tissue to the lumen and to diarrhoea. The severity of infection was determined by ability of the virus to propagate and destroy cells faster than the mucosa could replace the essential mature and functional cells. Factors which can slow this process of damage (smaller infectious dose, presence of immune factors, generation of non-specific factors, e.g. interferon, or poorly adapted virus), would benefit the host and lead to recovery or even to asymptomatic infection. Whatever the factors involved ultimately in the elimination of rotavirus from the host, the lack of mature small intestinal enterocytes suitable for virus growth must be one of them.

In comparison with the parent wild-type strain the "growth curve" of the attenuated strain was prolonged to at least six days, and the number of infected

small intestinal cells at any one time was greatly reduced. Loss of mature enterocytes was never sufficient to pose a threat to levels of digestive enzymes or absorption, as was evident from the lactase measurements and from the minimal changes observed histologically.

In some piglets infected with the attenuated strain there were regions or foci in which cells were extensively infected but the rest of the small intestine appeared unaffected and was probably capable of compensating for any appreciable regional loss of function. Undamaged areas contained sufficient numbers of mature enterocytes to sustain virus replication even 6 days post-infection. However during this time evidence of replication decreased, presumably due to the emergence of non-specific inhibitors and to onset of immune processes.

With both parent and attenuated strains the proximal half of the small intestine was the most consistently and intensely infected region although the pattern of infection from animal to animal was far from uniform, even in animals from the same litter given the same dose and strain, and killed at the same time. The difference was also manifested clinically which may account for differences normally observed among individuals.

This study demonstrates that with the two porcine rotavirus strains used, attenuation was associated with a reduced rate of infection of enterocytes over time. Reduction in the rate of cell destruction led to minimal mucosal changes with little or no physiological and clinical consequences. Fully attenuated or avirulent strains will have little or no ability to replicate within the host. The immunizing potential of such strains would depend entirely on the quantity and antigenic integrity of virus necessary to stimulate the gut-associated lymphoid tissue. In such circumstances the need for more than one dose becomes essential. There is little evidence at present that any of the 3 rotaviruses of animal origin—two bovine and one rhesus—that have been tested as potential vaccines in humans [7, 8, 23] are capable of replication in vivo, having been extensively attenuated in vitro as well as being of a different host origin. Truly attenuated strains with limited capacity for replication are likely to be more successful vaccines, and viruses of human origin that have been attenuated or naturally occurring avirulent strains (e.g. "nursery strains") are likely to be more effective.

References

1. Tzipori S (1985) The relative importance of enteric pathogens affecting neonates of domestic animals. Adv Vet Sci Comp Med 29: 103–206
2. Wyatt RG, James HD, Pittman AL, Hoshino Y, Greenberg HB, Kalica AR, Flores J, Kapikian AZ (1983) Direct isolation in cell culture of human rotaviruses and their characterization into four serotypes. J Clin Microbiol 18: 310–317
3. Hoshino Y, Wyatt RG, Greenberg HB (1984) Serotypic similarity and diversity of rotaviruses of mammalian and avian origin as studied by plaque reduction neutralization. J Infect Dis 149: 694–702
4. Bridger JC, Pocock DH (1986) Variation in virulence of bovine rotaviruses. J Hyg 96: 257–264

5. Institute of Medicine (1986) Prospects for immunizing against rotavirus. In: New vaccine development: establishing priorities, vol 2, diseases of importance in developing countries. National Academy Press, Washington, DC, D-13-1-12

6. Midthun K, Greenberg HB, Hoshino Y, Kapikian AZ, Chanock RM (1985) Reassortant rotaviruses as potential live rotavirus vaccine candidates. J Virol 53: 949–954

7. Clark HF, Furukawa R, Bell LM, Offit PA, Perrella PA, Plotkin SA (1986) Immune response of infants and children to low-passage bovine rotavirus (strain WC3). Am J Dis Child 140: 350–356

8. Fernelius AL, Ritchie AE, Classick LG, Norman JO, Mebus CA (1972) Cell culture adapation and propagation of a reovirus-like agent of calf diarrhea from a field outbreak in Nebraska. Arch Ges Virusforsch 37: 114–130

9. Vesikari T, Isolauri E, D'Hondt E, Delem A, Andre FE, Zissis G (1984) Protection of infants against rotavirus diarrhoea by RIT 4237 attenuated bovine rotavirus strain vaccine. Lancet 1: 977–981

10. Woode GN, Bew ME, Dennis MJ (1978) Studies on cross-protection induced in calves by rotaviruses from calves, children and fowls. Vet Rec 103: 32–34

11. Mebus CA, Wyatt RG, Sharpee KS, Sereno MM, Kalica AR, Kapikian AZ, Twiehaus MT (1976) Diarrhoea in gnotobiotic calves caused by reovirus-like agent of human infantile gastroenteritis. Infect Immun 14: 471–479

12. Tzipori S, Makin T, Smith M (1980) The clinical response of gnotobiotic calves, pigs and lambs to inoculation with human, calf, pig and foal rotavirus isolates. Aust J Exp Biol Med Sci 58: 309–318

13. Bishop RF, Barnes GL, Cipriani E, Lund JS (1983) Clinical immunity after neonatal rotavirus infection. A prospective longitudinal study in young children. N Engl J Med 309: 72–76

14. Chrystie IL, Totterdell B, Banatvala JE (1978) Asymptomatic endemic rotavirus infections in the newborn. Lancet 1: 1176–1178

15. Perez-Schael I, Daoud G, White L, Urbina G, Daoud N, Perez M, Flores J (1984) Rotavirus shedding by newborn children. J Med Virol 14: 127–136

16. Tzipori S, Williams IH (1978) Diarrhoea in piglets inoculated with rotavirus. Aust Vet J 54: 188–192

17. Smith M, Tzipori S (1979) Electrophoresis of rotavirus RNA derived from 6 different animal species. Aust J Exp Biol Med Sci 57: 583–585

18. Albert MJ, Unicomb LE, Tzipori S, Bishop RF (1987) Isolation and serotyping of animal rotaviruses and antigenic comparison with human rotaviruses. Arch Virol 93: 123–130

19. Makin T, Tzipori S (1980) Inexpensive technique for the production and maintenance of gnotobiotic piglets, calves and lambs. Aust Vet J 56: 353–359

20. Tzipori S, Robins-Browne RM, Gonis G, Hayes J, Whithers M, McCartney E (1984) Enteropathogenic Escherichia coli enteritis: evaluation of the gnotobiotic piglet as a model of human infection. Gut 26: 570–578

21. Coulson BS, Unicomb LE, Pitson GA, Bishop RF (1987) Simple and specific enzyme immunoassay using monoclonal antibodies for serotyping human rotaviruses. J Clin Microbiol 25: 509–515

22. Dahlquist A (1964) Method for assay of intestinal disaccharidases. Anal Biochem 7: 18–25

23. Anderson EL, Belshe RB, Bartram J, Crookshanks-Newman F, Chanock RM, Kapikian AZ (1986) Evaluation of rhesus rotavirus vaccine (MMU 18006) in infants and young children. J Infect Dis 153: 823–831

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