The Genomes of Rotaviruses Isolated from Chronically Infected Immunodeficient Children

By STEVE PEDLEY, FIONA HUNDLEY, IAN CHRISTIE, MALCOLM A. MCCRAE and ULRICH DESSELMERGER

1 Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, 2 Institute of Virology, University of Glasgow, Church Street, Glasgow G11 5JF and 3 Department of Virology, St Thomas’s Hospital, London SE1 7EH, U.K.

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SUMMARY

Abnormal RNA migration profiles were found in numerous rotavirus samples from two chronically infected children suffering from severe combined immunodeficiency. In both cases additional bands consisting of dsRNA were found migrating between RNA segments 1 and 7, and in one case RNA segment 11 was lost from the profile. Hybridization studies using segment-specific cloned cDNA probes indicated that some of the additional bands had sequence homologies with normal rotavirus dsRNA segments. In most cases these sequences were derived from genome segments of lower molecular weight by the formation of covalently linked concatemers.

INTRODUCTION

Rotaviruses have been recognized as the main cause of acute viral gastroenteritis in infants and young animals of a wide variety of species (Flewett & Woode, 1978; McNulty, 1978). The rotavirus infection is normally acute with diarrhoea lasting from a few days up to a week, during which time large numbers of rotaviruses can be detected in the faeces. Recovery of a child from diarrhoea is associated with the cessation of virus shedding from the faeces and with development of a seroconversion in rotavirus-specific antibody (Gurwith et al., 1981).

Immunodeficiency syndromes in man, in particular severe combined immunodeficiency (SCID), are frequently associated with severe and chronic virus infections during the first year of life. If the gut is involved, the clinical symptoms are protracted diarrhoea (PD) and the children fail to thrive (McConnell et al., 1981; Hayward, 1982). Several cases of prolonged excretion of rotaviruses associated with PD have been described in children with immunodeficiencies (Saulsbury et al., 1980; Booth et al., 1982; Chrystie et al., 1982). The shedding of rotaviruses from these chronically infected children can last from several weeks to many months. As well as rotaviruses, other viruses (adenoviruses, caliciviruses, astroviruses) have been found in the faeces simultaneously and also for a prolonged period of time (Chrystie et al., 1982).

Rotaviruses have a double-stranded RNA genome consisting of 11 segments which can be easily isolated from the virus and separated by PAGE. Recently, a highly sensitive technique has become available for visualizing RNA segments in gels by silver staining (Herring et al., 1982; Follett & Desselberger, 1983a). We have examined multiple faecal specimens obtained from two children with SCID for the presence of the RNAs of the rotavirus genome. Abnormal rotavirus RNA profiles were obtained which were characterized by the appearance of additional bands of dsRNA in both cases and by the loss of one segment from its original position in one case. Hybridization studies using cloned rotavirus cDNAs as segment-specific probes (McCrae & McCorquodale, 1982) showed that some of the extra bands contained viral sequences derived from rotavirus genome segments. The biological significance of this finding is discussed.
METHODS

Viruses. Seventy-eight faecal specimens of child U.H., obtained from May 1980 to April 1981, and 23 faecal specimens of child A.K., obtained from January 1982 to May 1983, were analysed.

The case history of child U.H. has been the subject of several publications (Christie et al., 1982; Booth et al., 1982). In brief, the child, a male, developed PD at 2 months of age, and an SCID was diagnosed. Rotaviruses were detected by electron microscopy (EM) in many faeces over a period of 8½ months. Prolonged excretion of adenoviruses, calciviruses, astroviruses and small round viruses was also observed. A bone marrow transplant was performed at 12 months of age but this was unsuccessful, and the child died 2 months later from a disseminated adenovirus infection.

A.K., a female born in September 1981, presented with severe pneumonia and septicaemia at the age of 7 weeks. This resolved under intensive antibiotic treatment. The symptoms of profound lymphopenia, low serum immunoglobulin levels and the absence of palpable lymph nodes and of a thymus shadow on chest X-ray suggested the diagnosis of SCID. This was confirmed by the findings of extremely low numbers of T lymphocytes, of complete absence of T lymphocyte function and of a lack of the enzyme adenosine deaminase. Treatment of this condition was initially with exchange blood transfusions. In January 1982, food intolerance and persistent diarrhoea developed and the child failed to thrive. Examination of the faeces by EM demonstrated rotavirus particles which were excreted during the following 7 weeks. In March, a bone marrow transplant (donated by the mother) was carried out. The grafting proved to be successful in that the number or circulating T cells increased and that T cells became functional 4 months after the transplant. Unfortunately, at this time an autoimmune haemolytic anaemia developed which required treatment with high doses of steroids. A number of infections after the bone marrow transplant (pneumonia in August 1982, chickenpox in October 1982 and a septicaemia due to Klebsiella in December 1982) were overcome. Multiple food intolerance persisted but rotavirus was not found between March 1982 and May 1983 in routine examinations of monthly faecal specimens (by EM). Re-admission to hospital in May 1983 with the symptoms of acute gastroenteritis revealed the presence of rotaviruses in only one faecal specimen (by EM) but the child recovered within 1 week under symptomatic treatment.

Bovine rotavirus (U.K. Compton strain), grown in BSC-1 cells, was used as a control in several experiments.

Virus purification. All faecal specimens were suspended to 10% (w/v) in phosphate-buffered saline (PBS), extracted by vigorous shaking and pipetting, and centrifuged at low speed. Aliquots of the supernatants were tested serologically as outlined below. Virus was pelleted from the supernatants by ultracentrifugation for 1 h at 110000 g and 4 °C through a 30% sucrose cushion. The pellets were resuspended in a small volume of PBS and aliquots taken for EM.

Bovine rotavirus was purified from the supernatants of infected tissue cultures following the same procedure.

Electron microscopy. Faecal extracts (concentrated by ultracentrifugation) were negatively stained with 3% phosphotungstic acid pH 6.8, and examined in a Philips 201C electron microscope.

RNA extraction, gel electrophoresis and silver staining of gels. The viral RNA was extracted from the virus suspension of the pellet after ultracentrifugation and the segments were separated by electrophoresis on 2.8% polyacrylamide-6 M-urea slab gels as described previously (Follett & Desselberger, 1983a). Silver staining of gels was as described (Herring et al., 1982; Follett & Desselberger, 1983a). Molecular weights of RNA segments and of additional RNA bands of patients' specimens were determined from silver-stained gels by using the molecular weights of co-electrophoresed bovine rotavirus RNA segments (Rixon et al., 1984) as standards.

Comparison of viral RNA migration patterns under denaturing and non-denaturing conditions. Viral RNA was extracted from 0.5 to 1.0 cm³ of faeces with phenol and partially purified by differential precipitation with 2 M- and 4 M-LiCl according to the method of Clarke & McCrae (1981). The RNA was 3' end-labelled with cytidine 3',5'-[32P]-bisphosphate ([32P]pCp) and fractionated on 7.5% polyacrylamide gels using the discontinuous system of Laemmli (1970) as previously described (Clarke & McCrae, 1981). Viral dsRNA was fractionated under denaturing conditions by electrophoresis on 1.5% agarose gels containing 5 mM-methylmercuric hydroxide at 18 mA for 16 h (Bailey & Davidson, 1976).

Testing for the presence of dsRNA. RNAs of selected samples were treated with different restriction endonucleases (BamHI, HindIII; Bethesda Research Laboratories) and with ribonuclease T1 (Sankyo; from Calbiochem) with and without prior denaturation (90 s boiling in 5 µl, followed by quick chilling in an ice bath). Published procedures were used for digestion with restriction endonucleases (Mantalis et al., 1982) and with ribonuclease T1 (Pedersen & Haseltine, 1980). The analysis of digests was done by PAGE followed by silver staining (Follett & Desselberger, 1983a).

Hybridization experiments. Unlabelled, partially purified dsRNA of the rotavirus genome was fractionated on 7.5% polyacrylamide gels as described above and visualized by staining with ethidium bromide. The RNA was denatured and partially degraded by soaking the gel in 0.1 M-NaOH at room temperature for 15 min and then electrophoretically transferred to diazo-2-aminophenylthioether (DPT) paper according to the method of Street et al. (1982), using a Bio-Rad Trans-Blot Cell.

The DPT papers were prepared for hybridization at 26 °C in prehybridization buffer [50% formamide, 5 × SSC

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buffer (1 × SSC: 150 mm-sodium chloride, 15 mm-sodium citrate, pH 7-4), 50 mm-phosphate buffer pH 7-5.
130 µg/ml denatured salmon sperm DNA, 0·1% each of bovine serum albumin (BSA), polyvinylpyrrolidone
(PVP) and Ficoll, and 0·1% glycine] for 8 to 12 h. Hybridization was performed using nick-translated probes
(Rigby et al., 1977) of cloned cDNA copies of the genome segments of the U.K. tissue culture-adapted calf rotavirus
(McCrue & McCorquodale, 1982) in hybridization buffer (4 parts prehybridization buffer containing 0·02 %
each of BSA, PVP and Ficoll; plus 1 part 50% dextran sulphate) at 26 °C for 18 to 24 h. The DPT papers were
washed for 5 min each at room temperature in four changes of 2 × SSC containing 0·1% SDS, and for 15 min each
in two changes of 0·1 × SSC plus 0·1% SDS at 36 °C. These conditions were established to show sequence
homologies of greater than 70%; Hybridization was monitored by autoradiography of the blots.

Serological analysis. Viruses were tested in the Rotazyme test (Abbott Laboratories, North Chicago, Ill.,
U.S.A.; Beards & Bryden, 1981) for the presence of the common group A-specific antigen of mammalian viruses
(Pedley et al., 1983) and were subgrouped by using an ELISA test (Thouless et al., 1982; Greenberg et al., 1983;
Follett et al., 1984).

RESULTS

Genome profiles (RNA migration patterns) were established of rotaviruses obtained from the
faeces of both patients (A.K. and U.H.). A.K. shed rotaviruses over a period of 7 weeks (7
positive faeces), and from patient U.H. rotaviruses were obtained over a period of 8½ months (in
42 out of 78 faecal specimens). The rotaviruses of both patients belonged to subgroup 2
(Thouless et al., 1982) of human group A (Pedley et al., 1983) rotaviruses.
The profiles obtained from most of the samples of patient A.K. are shown in Fig. 1 (a).
Genomic RNAs of bovine rotavirus (U.K. tissue culture-adapted Compton strain) and of
human rotaviruses obtained from an acute infection were used as controls. It was observed (Fig.
1 a) that in addition to the usual 11 RNA segments (here almost co-migrating with those of the
human control) extra bands of various intensities migrated between segments 4 and 5 and also
(although showing faintly) between segments 1 and 2 in the three later isolates (indicated by
arrows).

By digesting the genomes of those rotaviruses showing additional bands in genome profiles
with ribonucleases (with and without prior denaturation by heat and chilling) and by deoxyribo-
nucleases it was established that the additional bands observed consisted of dsRNA (results not
shown).

(a) (b)

Fig. 1. RNA profiles of rotavirus samples from patient A.K. Genomic dsRNA was extracted from
faeces and 3 end-labelled with [32P]pCp as described (Clarke & McCrae, 1981). Samples were analysed
on either a 7·5% non-denaturing polyacrylamide gel (a) or a 1-5% agarose gel containing 5 mM-methyl-
mercuric hydroxide (Bailey & Davidson, 1976) to denature the RNAs (b). 3 end-labelled RNAs of
bovine rotavirus and of a human rotavirus obtained from an acute infection served as internal controls.
Segments are numbered at both sides of the gels and additional bands are indicated by arrowheads.
In order to determine whether or not the additional bands were the result of secondary structure variation and/or non-covalent linkage of RNA segments, we analysed samples in denaturing methylmercuric hydroxide-agarose gels (Bailey & Davidson, 1976). An experiment performed with the samples of patient A.K. (Fig. 1a) is shown in Fig. 1(b). Although the resolution achieved is inferior to that obtained in polyacrylamide gels (Fig. 1a), at least one of the additional bands between RNA segments 4 and 5 was still visible.

From their migration on polyacrylamide gels, it seemed possible that the additional bands of dsRNA were derived from genome segments seen in standard rotavirus RNA profiles. In order to confirm this and to determine the origin of the additional bands, their sequence relationship to the genome segments of normal group A rotaviruses was studied. The viral dsRNA was fractionated on polyacrylamide gels, electrophoretically transferred to DPT paper and hybridized to nick-translated cDNA clones of a bovine rotavirus genome (McCrae & McCorquodale, 1982) using conditions that would detect sequence homologies of greater than 70% and which gave segment-specific reactions in the controls. The results obtained with the samples from patient A.K. are shown in Fig. 2. The probe to RNA segment 2 hybridized to a segment co-migrating with segments 2 of both the bovine and human controls, and to a second band migrating just ahead of RNA segment 4 of the labelled bovine rotavirus control RNA (Fig. 2a). Hybridization to a third band was seen with the sample taken on 8.3.82 (Fig. 2a). The probe to RNA segment 9 hybridized to a segment that co-migrated with segments 7, 8 and 9 of the bovine and human controls and to a well-separated triplet of RNA species migrating above the RNA segment 5 of the labelled bovine rotavirus control RNA (Fig. 2c). This triplet of bands was evident on the original profile analysis of the samples (Fig. 1a). The most complex pattern of hybridization was seen for the segment 6-specific cDNA probe (Fig. 2b). In all the A.K. samples analysed, this probe hybridized to a segment that co-migrated with the human segment 6. Between 11.2.82 and 17.2.82, hybridization occurred to two additional bands that migrated in the RNA segment 4 region of the labelled bovine rotavirus control RNA. However, in the sample 2.3.82, the slower migrating of these extra bands was lost, but reappeared in the last two samples accompanied by a third band migrating ahead of the others. Some of the extra bands (Fig. 2a, b) migrated in very close proximity to RNA segments 2, 3 and 4 but could be differentiated on silver-stained gels of the samples (not shown). cDNA probes specific for RNA segments 5, 7, 8 and 10 were also used in this analysis (data not shown) but they either failed to cross-hybridize with the A.K. samples (segment 5-specific probe) or hybridized to only a single segment which co-migrated with the bovine rotavirus RNA segment corresponding to the probe. Furthermore, RNA samples of patient A.K. were fractionated on a denaturing methylmercuric hydroxide gel, electrophoretically transferred (transblotted) and the blots hybridized to a nick-translated cDNA probe which previously had hybridized with one or more of the additional bands. An example is given in Fig. 2(d). It can be seen that samples which were transblotted from a denaturing gel reacted with segment 9-specific probe to give a complex reaction pattern (Fig. 2d) virtually identical to that obtained with samples transblotted from a conventional gel (Fig. 2c). These results indicate that the additional bands of dsRNA contained segment-related sequences in the form of covalently bonded concatemers.

A similar study was performed on the genomes of selected samples from patient U.H. Fig. 3 shows the genome profiles of a representative selection of samples obtained from this patient over a period of 8½ months. Two abnormalities were observed. (i) In addition to the normal RNA segments, extra bands were found over a wide range of the profile (between RNA segments 2 and 7, labelled by arrowheads). The extra bands varied in intensity and appeared and disappeared on passing through the chronological series. (ii) RNA segment 11 was only seen in the first isolate of 28.5.80, and there only weakly (see below); it then disappeared.

No additional bands migrating faster than the smallest segment were revealed by short duration electrophoresis of the RNAs of these specimens (results not shown).

RNAs of selected isolates of patient U.H. (Fig. 4a) were separated on methylmercuric hydroxide-containing gels which showed no change in the position of many of the additional bands (Fig. 4b). Samples were blotted from a polyacrylamide gel onto DPT paper and then hybridized to segment-specific cDNA probes (as for Fig. 2). The observations obtained from
Human rotavirus genomes

Fig. 2. Hybridization analysis of rotavirus RNA samples from patient A.K. Viral dsRNA was fractionated either on 7.5% polyacrylamide gels (a to c) or on a 1.5% agarose gel containing methylmercuric hydroxide (d) and transferred to DPT paper as described under Methods. The transblots were then probed with nick-translated cloned cDNAs specific for RNA segments 2 (a) 6 (b) and 9 (c, d) of U.K. strain rotavirus (McCrae & McCorquodale, 1982). 3' end-labelled bovine (L Bovine) rotavirus dsRNA and unlabelled bovine and human rotavirus control RNAs were electrophoresed and blotted in parallel to serve as markers. Lanes 3 and 4 of (a) and (b) show some background hybridization.

this analysis of the samples of patient U.H. were essentially the same as those obtained in the material from patient A.K.: the probes either cross-hybridized to a single band that co-migrated with the human and bovine RNA segments corresponding to the probe (e.g. segment 2-,
segment 5-, and segment 7-specific probes), or they cross-hybridized to the corresponding segment and additional bands of dsRNAs of higher molecular weight. Thus, the segment 6-specific probe cross-hybridized to the corresponding segment and to one band higher up in the gel (Fig. 4c). In the case of hybridization to the segment 8-specific probe (Fig. 4d), the migrational position of the cross-hybridizing extra bands varied during the course of infection. It should be noted that at present the segmental origin of some of the additional bands remains to be elucidated.

The molecular weights of additional bands whose segment derivation was determined were not found to be simple multiple integers of the molecular weights of the RNA segments from which they were derived. Thus, the apparent molecular weight of the additional band cross-hybridizing with the segment 6-specific probe was 1.86 times the molecular weight of segment 6.
Fig. 4. Hybridization analysis of rotavirus RNA samples obtained from patient U.H. (a) 3' end-labelled genomic dsRNA separated on a 7.5% non-denaturing gel. (b) The same samples as shown in (a) fractionated on a 1.5% agarose gel containing methylmercuric hydroxide. (c, d) Unlabelled viral dsRNA fractionated on a 7.5% polyacrylamide gel and transferred electrophoretically to DPT paper. The transblots were hybridized to nick-translated cDNA probes specific for segment 6 (c) or for segment 8 (d). Segment numbers are indicated to both sides of the gels.

(Fig. 4c), and the molecular weights of the bands cross-hybridizing with the segment 8-specific probe were 1.86 and 1.53 times higher than the molecular weight of segment 8 (Fig. 4d).

The virus particles from both these chronic infections appeared to be normal when viewed by EM. Therefore, an incorporation of extra segments within the virions might be expected to be accompanied by a compensating loss in the amounts of normal genomic RNA segments. Since the binding of silver by nucleic acid in the silver staining technique occurs stoichiometrically (Whitton et al., 1983), this possibility was investigated by densitometry (Whitton et al., 1983) of silver-stained genome profiles (Fig. 3). Taking the amount of RNA segment 10 as unity, it was found that the amounts of RNA present in segment 6 and in the triplet of segments 7 to 9 (segment 6- and segment 8-related sequences were present in large additional bands, see above) were lower than expected from normal profiles. RNA segment 11 decreased in the relative amount present in sample U.H. of 28.5.80 (Fig. 3) before it disappeared. The total amount of all
additional bands increased steadily over time, up to 33.9% of the whole RNA present (results not shown).

Finally, it is of interest to report that the RNA of rotavirus which was isolated from a single faecal specimen of patient A.K. in May 1983 (1 year after the chronic infection and after a successful bone marrow transplantation) did not contain additional bands (data not shown). Clinically, this was an acute rotavirus infection and was overcome within a week.

**DISCUSSION**

This report deals with the characterization of rotaviruses obtained from two chronic infections in man. In both cases the severely compromised immune system of the patients resulted in a failure to clear rotavirus from the body after 4 or 5 days as would normally be seen in acute rotavirus infections. Consequently, both children suffered from PD and shed virus in the faeces for 7 weeks in one case and for 8½ months in the second case. Genome profiles of multiple rotavirus samples obtained from these patients differed from genome profiles normally seen in acute infections (Follett & Desselberger, 1983a; Follett et al., 1984) in several ways. (i) Additional bands of dsRNA migrating between segments 1 and 7 to 9 were found in both cases, varying in quantity and changing their pattern during the course of infection. (ii) In one case (U.H.) RNA segment 11 which was clearly present at the beginning of the infection rapidly disappeared from its normal position as the infection proceeded.

Studies of hybridization to Northern blots of rotavirus RNAs using cloned cDNA probes specific for single rotavirus RNA segments showed that some of the additional bands of dsRNA contained sequences which were derived from normal rotavirus RNA segments. So far, we have not elucidated the segmental origin of all additional bands seen. The extra bands containing viral sequences were in the main not derived by deletions from larger RNA segments but had emerged by some form of concatemerization of smaller genome RNAs. Analysis of the samples under completely denaturing conditions revealed that this concatemerization resulted from covalent linkage of RNAs rather than from non-covalent joining. Measurement of the migration distances of the extra bands showed that their molecular weights were not simple multiple integers of the molecular weights of the normal RNA segments from which they were derived. Finally, quantification of silver-stained RNA profiles demonstrated that, concomitant with the appearance of additional bands carrying rotavirus-specific RNA sequences, there was a decrease in the relative amounts of normal RNA segments from which the additional bands were derived. Thus, it is conceivable that part of the RNA genome occurred in an abnormal configuration within single virus particles or that a mixed population of viruses possessing normal and abnormal genomes co-existed. Only when propagation of these isolates has been achieved in vitro will it be possible to resolve this problem.

The abnormal genomes of rotaviruses generated during chronic infection raised the question of whether or not they represent defective interfering (DI) particles (Holland et al., 1980, 1982) which in this case had occurred naturally. DI particles of other viruses, artificially created in the laboratory by passaging at high multiplicity of infection, contain shortened genomes, in contrast to the observation presented here. In the case of viruses possessing segmented RNA genomes the generation of DI particles is associated with the loss of normal large RNA segments and the appearance of small RNAs (DI RNAs), mostly migrating faster than the smallest normal RNA segment on polyacrylamide gels (Davis et al., 1980; Brown et al., 1983). The DI RNAs are generated either by internal or terminal deletions from the larger RNA (Davis et al., 1980; Holland et al., 1980) or by the formation of mosaic structures containing sequences from more than one of the larger RNA segments (Fields & Winter, 1982). The migrational pattern of the additional bands seen in the present study eliminates the possibility of them having emerged by deletions. Studies to investigate whether or not they are mosaic structures are in progress.

So far, it has not been possible to adapt the viruses under study to growth in tissue culture; as a consequence we have not been able to investigate whether these viruses exhibit some of the biological properties normally associated with DI virus populations. However, studies carried out on passages at high multiplicity of infection of tissue culture-adapted bovine rotavirus have shown that this in vitro procedure also generates viruses with abnormal genome profiles in which
some of the normal RNA segments have disappeared and additional bands of dsRNA related in sequence to the disappeared segments and of higher molecular weight were detected (F. Hundley & U. Desselberger, unpublished observations). Detailed analysis of these observations is currently in progress. It will be of interest to see whether this phenomenon indicates a generally applicable rapid method for introducing a large number of mutational changes into the genome of dsRNA viruses. For both the human rotavirus obtained from patients with chronic infection and the bovine rotaviruses passed at high multiplicity of infection it is conceivable that a 'DI selection effect' (Holland et al., 1982) could have been operative. Most of the patients' specimens did not contain enough RNA to produce serial oligonucleotide maps (Follett & Desselberger, 1983b) which would allow an analysis of the frequency of mutations.

Viruses obtained from serial samples of one of the patients had lost genome segment 11 from its original position. Rotaviruses apparently lacking segment 11 were recently isolated from man (Rodger et al., 1982) but they did not share the common group-specific antigen of human and mammalian group A (Pedley et al., 1983) rotaviruses. A new subgroup similar to those observed in pigs (Bridger et al., 1982) and chickens (McNulty et al., 1981) was proposed (group C rotaviruses; Pedley et al., 1983). The viruses described in this report shared the common group-specific antigen of human rotaviruses (group A antigen; Pedley et al., 1983) and were of subgroup 2.

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