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Unusual Indian asymptomatic neonatal human rotaviruses: Potential candidates for development of live reassortant rotavirus vaccines

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Abstract

We have isolated a large number of unusual rotavirus strains from asymptomatically infected new-born children from hospitals in Bangalore during a 3-year period from 1988 to 1991. About 36 per cent of the neonates in the age group 2 to 60 days showed asymptomatic infection, exclusively by these novel strains. All the isolates, without exception, showed 'long' RNA pattern but for subgroup I specificity which is characteristic of animal rotaviruses. Serotypic analysis revealed that these viruses did not belong to any known human serotypes, instead, were related to bovine serotype 10 viruses. Genogroup analysis showed a high level of relatedness to serotype 10 bovine rotavirus and a low level of homology to human serotype 1 virus. Some of these strains have been adapted to tissue culture. Because of their attenuated nature and their ability to grow to high titres in cell culture and to effectively infect humans, these asymptomatic neonatal Indian strains represent ideal candidates towards development of reassortant live rotavirus vaccines as an alternative to recombinant vaccines. I321 and related neonatal viruses represent ideal candidates to study not only the molecular evolution of human rotaviruses but also the mechanism of virulence and host range restriction.

Key words: Human rotaviruses, gastroenteritis, vaccines.

1. Introduction

Rotavirus is the major causative agent of acute infectious gastroenteritis in the young of many species including humans and a variety of wild and domestic animals[1]. It is the leading cause of childhood morbidity and mortality in developing countries, accounting for about 2 million deaths annually[1]. It also causes disease in adults and immuno-compromised individuals[1]. Rotavirus belongs to the family *Reoviridae* which comprises nonenveloped, icosahedral viruses with double-shelled protein capsid and segmented, double-stranded RNA genome[1,2]. Rotavirus genome consists of 11 double-stranded RNA segments ranging in size from 663 to 3,302 base pairs and each segment is monocistronic encoding a single protein[2]. Except for gene 4-

and 9- encoded proteins VP4 and VP7, respectively, the functions of other gene products is not well understood[2]. Rotavirus has been classified into 7 groups, A to G, based on group-specific antigens detected primarily on VP6 by immune electron microscopy and ELISA[3,4]. Group A rotaviruses are the subject of extensive studies as they constitute the majority of rotaviruses in man. Rotaviruses exhibit two serological specificities, *i.e.*, serotype and subgroup[1,5–7]. The two outer capsid proteins, VP4, encoded by gene 4 and VP7, encoded by gene 9, determine two distinct serotype specificities, the P type and G type, respectively[1]. To date, at least 14 G and 10 P serotypes have been identified[1,5,8,9]. VP6, the inner capsid protein, encoded by gene 6, determines the subgroup specificity and at least two distinct specificities, subgroup I and II, have been identified[6,10].

Group A rotaviruses have also been classified as those having either 'long' or 'short' RNA electropherotype depending on the fast or slow migration of RNA segment 11, respectively[1, 11–13], on polyacrylamide gels. Recently, strains with 'super short' RNA pattern in which the tenth segment migrates slower than that of 'short' RNA pattern have been identified[14,15]. Vast majority of human rotaviruses with subgroup I specificity have a 'short' or 'super short' electropherotype and those with subgroup II specificity have 'long' RNA pattern[1, 12, 14]. Also, human rotavirus strains with serotype 2 specificity exhibit short RNA electropherotype and those belonging to serotypes 1, 3 and 4 have long RNA electropherotype[16,17]. But great majority of animal viruses exhibit long RNA pattern and subgroup I specificity[1, 6,18, 19]. In humans, only rotavirus serotypes 1, 2, 3 and 4 have been mostly encountered.

Rotaviruses have also been classified into genogroups on the basis of overall gene homology. Members of a genogroup share high degree of genetic relatedness with each other, but are significantly less homologous to members of other genogroups[20, 21]. In general, rotaviruses from different species have been observed to belong to species-specific genogroups. Majority of human rotaviruses fall into three distinct genogroups, (i) Wa-like, (ii) DSI-like, and (iii) AU-1-like[22].

In recent years, a few human rotavirus strains exhibiting subgroup I specificity, but 'long' RNA pattern, have been isolated from patients suffering from gastroenteritis[23,24,25]. These unusual strains possess either a known or a new serotype[26–28] specificity and appear to be genetically related to strains derived from animals such as cats and dogs[19]. Human rotavirus isolates exhibiting subgroup I specificity and long RNA pattern have been postulated to be of animal origin[6].

Rotavirus infections, both symptomatic and asymptomatic, have also been observed in new-born children[1,27,29–31]. These neonatal rotavirus infections differ from those of older children in clinical and epidemiological features. Neonatal infections, in most cases, are asymptomatic, occur throughout the year and generally have electropherotypes distinct from those infecting older children in the community at the same time[27]. The lack of virulence in majority of the neonatal isolates does not appear to be related to a specific serotype as each of the four major human serotypes have been isolated from asymptomatically infected neonates[26].

Table I

Incidence of asymptomatic neonatal infections and characterization of the isolates

Number of samples analysed	370
Number positive for rotaviral RNA	133
Per cent positive	36
Subgroup I +ve	133
Subgroup II +ve	–
Long electropherotype	133
Short electropherotype	–

We have isolated several rotavirus strains from asymptomatic newborns at hospitals and clinics in Bangalore. All the neonatal strains, without exception, showed subgroup I specificity and long RNA electropherotype[30]. Some of these strains were adapted to tissue culture and by serotype analysis, surprisingly, were observed to be related to serotype 10 bovine rotaviruses[18]. This finding was further confirmed by geno-group analysis which showed high degree of homology to bovine serotype 10 rotavirus KK3[18].

We have also studied the molecular epidemiology of rotavirus infection in younger children, suffering from gastroenteritis, in the age group between 6 months and 7 years. The genes encoding the inner and outer capsid proteins, VP6, and VP4 and VP7, respectively, from the predominant serotypes have been cloned and are being expressed towards development of reagents for recombinant rotavirus vaccines.

2. Unusual rotaviruses in asymptomatic neonatal infections

Clinical fecal samples from 2- to 60-day-old asymptomatic neonates as well as young children suffering from gastroenteritis were collected from six major hospitals and clinics in Bangalore. Samples positive for the presence of rotavirus RNA were detected by polyacrylamide gel electrophoresis (PAGE) of the extracted RNA and detection of 11 double-stranded RNA (dsRNA) segments characteristic of rotavirus genome by silver staining technique[11,30]. We observed a 36 per cent incidence of asymptomatic rotavirus infection in newborn children (Table I). All the strains exhibited long RNA pattern (Fig. 1) and subgroup I specificity[30]. This observation pointed to the unusual nature of these virus strains as such viruses are found only in animals[6]. Serotype analysis with available serotype-specific monoclonal antibodies (mAbs) indicated that these viruses did not belong to any known human rotavirus serotypes 1, 2, 3, 4, 8, 9 and 12[18, 30]. Infection of the neonates by these unusual viruses was exclusive as we did not observe viruses belonging to other human serotypes in neonatal infections.

3. Serotype analysis of neonatal viruses

Serotype and subgroup specificities of the neonatal strains were determined as previously described[20, 30]. A few asymptomatic neonatal isolates I195, I321 and I422 were

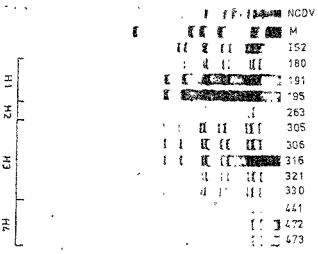

FIG. 1. RNA electrophoretic analysis of some of the positive asymptomatic neonatal isolates. RNA segments were separated by electrophoresis on 10 per cent polyacrylamide gel for 24 h and stained with silver nitrate. The numbers above the lane represent the code numbers of the samples collected from different hospitals H1 to H4 represent the code names given to the hospitals M stands for DNA molecular weight standards containing Hind III-digested bacteriophage Lambda DNA and Hae III-digested $\phi \times 174$ RF DNA. IS2 is an Indian rotavirus strain, isolated from a patient, exhibiting subgroup I and serotype 2 specificity and short RNA electropherotype.

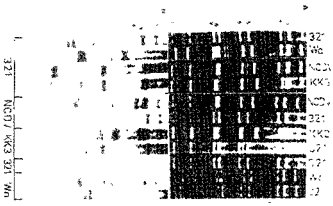

FIG. 2. Hybridization patterns obtained from ^{32}P-labeled ssRNA probes to the genomic RNA from various mammalian rotavirus strains indicated on the top of the lanes. A. Ethidium bromide stained gels under uv illumination. B. Corresponding autoradiographs. The ^{32}P-labeled ssRNA probe strains are listed in the bottom of the lanes while the genomic RNA strains are indicated on the top of the lanes. RNA segments are indicated to the left of each panel.

adpated to tissue culture as previously described[32]. All the three strains showed long RNA pattern and subgroup I specificity as the parental strains had prior to cell culture adaptation[30]. After two passages in primary AGMK cells followed by two passages in MA104 cells, the plaque-purified neonatal strains I321 and I422 were subjected to serotype analysis by ELISA using monoclonal antibodies, directed against the outer capsid protein VP7, specific for different serotypes. As shown in Table II, I321 and I422 reacted strongly with serotype 10-specific mAbs and did not react with those specific for other rotavirus serotypes. These results suggested that

Table II

Serotype analysis of cultivated neonatal asymptomatic strains as determined by ELISA with serotype-specific monoclonal antibodies

Indian neonatal	*Monoclonal antibody (serotype specificity)*						
	5E8 (1)	*1C10 (2)*	*159 (3)*	*ST3 (4)*	*5B8 (5)*	*IC3 (6)*	*B223/N7 (10)*
I321	71	49	115	35	56	52	400
I422	102	53	165	32	63	44	642

Data shown as $A_{405} \times 1000$

Table III
Neutralization titer by focus reduction assay against the indicated viruses

Hyperimmune guinea pig antisera to strain I321	*Virus (serotype)*										
	Wa (1)	*S2 (2)*	*RRV (3)*	*ST3 (4)*	*OSU (5)*	*NCDV (6)*	*69M (8)*	*WI61 (9)*	*B223 (10)*	*L26 (12)*	*I321/ I422*
#1	<200	<200	800	3200	<200	800	<200	800	6400	<512	>12800
#2	<200	<200	<200	800	<200	200	<200	200	12800	ND	>12800

I321 and related strains belong to serotype 10 viruses that are observed only in cattle. We have also produced polyclonal antibodies in guinea pigs against I321 and were tested in a focus reduction assay against prototype viruses belonging to different serotypes. Again, the highest neutralization titre against serotype 10 bovine rotavirus strain B223 was observed (Table III) thus confirming the above results of serotype analysis[18, 30].

4. Genetic analysis of Indian neonatal viruses

To further confirm the serotype 10 specificity and to determine the genetic origin of these asymptomatic strains, genogroup analysis of I321 was carried out as described by Nakagomi and Nakagomi[20, 21]. Radiolabeled, single-stranded RNA transcribed *in vitro* from single-shelled virus particles was hybridized to different viral genomic RNAs in solution and the resulting hybrids were analysed by PAGE. As shown in Fig. 2, I321 showed a high level of homology with bovine serotype 10 rotavirus strain KK3, a medium-level homology to serotype 6 bovine rotavirus, NCDV, and a low-level of homology with human serotype 1 virus strain, Wa. The level of homology to other human or animal serotype rotaviruses was very low[18] (data not shown). Genes 4 and 9, encoding the outer capsid proteins VP4 and VP7, respectively, were derived from bovine serotype 10 virus. These studies not only confirmed the serotype 10 specificity of the neonatal strains but also suggested that these unusual strains might represent natural reassortants between a serotype 10 bovine rotavirus and a human rotavirus probably of serotype 1. Genogroup analysis also revealed that some of the nonstructural protein-encoding genes 5, 7 or 8 of I321 might have been derived from a human rotavirus rather than serotype 10 bovine rotavirus. The fact that I321 efficiently infects humans, in spite of having bovine serotype 10 rotavirus genome background, indicates that some of the nonstructural genes might determine pathogenicity and host range restriction.

5. Prospects for development of effective rotavirus vaccines

Diarrhoeal disease is one of the major causes of morbidity and mortality among infants and young children, especially in developing countries. As more than 40 per cent of gastroenteritis cases are thought to be caused by rotavirus infection in young children, development of an effective vaccine against rotavirus disease is of immense importance in formulating public health priorities. Two approaches can be used to develop effective vaccines against rotavirus disease.

i) Reassortant rotavirus vaccine based on a donor strain that grows well in cell culture and efficiently infects humans. This approach involves development of rotavirus strains containing VP4 and VP7 of the four major human rotavirus serotypes by replacement of genes 4 and 9 of the donor strain by genetic reassortment.

ii) Recombinant vaccine containing a cocktail of the inner and outer capsid proteins, VP6 and VP4, and VP7, respectively, from the four predominant human serotypes. Vaccines based on single or double antigens of the outer capsid might also be useful.

All rotavirus vaccines tested to date[1, 2] have failed in the trials because of the following reasons:

i) Human rotaviruses are difficult to grow in cell culture to high titres to produce vaccines and thus most of the vaccines are based on animal viruses.

ii) Majority of rotaviral vaccines were based on animal rotaviruses which due to host range restriction infect humans very poorly even at high doses and hence elicit poor immune response.

iii) Four rotavirus serotypes are predominantly observed in humans and thus a vaccine specific for one serotype was not effective against infection by other serotypes.

iv) Reassortant rotavirus vaccines were also not successful as the donor strains used for generating reassortants were of animal origin.

Because of the above-mentioned limitations associated with the existing experimental rotavirus vaccines, there is an urgent need to develop alternative comprehensive vaccines incorporating the major antigenic determinants from the four predominant human rotavirus serotypes. Development of reassortant rotavirus vaccine is based on the observation that when two different strains of a virus having segmented genome are coinfected, some progeny viruses containing one or more genes of other strain arise due to exchange of the genome segments. This phenomenon has been exploited for development of experimental rotavirus vaccines[1, 2, 33]. For a reassortant vaccine to be successful, the donor strain should be able to efficiently infect humans and grow in cell culture to high titres. A successful recombinant vaccine should include both antigenic proteins of the outer capsid from the four human serotypes. The fact that VP7 expressed in soluble form acts as a poor immunogen compared to that associated with VP4 on the viral surface[34, 35] or that expressed on the cell surface[36] suggests that interaction of VP4 and VP7 and probably of VP6 also is essential for the recombinant vaccine to be effective. Although this approach is expensive, it is still feasible. Our attempts are oriented in these two directions for developing vaccine reagents based on rotaviruses isolated in India.

6. I321 as donor strain for reassortant vaccines

I321 appears to be a logical choice for development of live reassortant rotavirus vaccine because of the following attributes: (i) it is an animal-derived, highly

attenuated, natural isolate of humans, (ii) replicates efficiently in humans, at least in neonates[30], and (iii) it has been adapted to cell culture and can be grown to high titres[18].

These characteristics make I321 an excellent donor strain for generating reassortant rotaviruses that contain the major antigenic protein VP7 or both the outer capsid proteins VP4 and VP7 from the four predominant human serotypes. A cocktail of reassortant rotaviruses containing the outer capsid proteins from the four human serotypes on I321 background could serve as an effective 'Jennerian'-type live vaccine.

7. Recombinant rotavirus vaccines

Since four serotypes were predominantly encountered in human infections, a recombinant subunit vaccine should ideally contain at least one of the outer capsid antigenic proteins from all the four serotypes. Recent studies indicate that the immunogenicity as well as reactivity to neutralizing antibodies of VP7 is enhanced when expressed on the viral or cell surface compared to that of soluble form[34–36]. These studies suggest that association of VP7 with VP4 elicits better protective immune response. Since there is a cosegregation specificity between VP4 and VP7, in nature, in different serotypes, a vaccine containing both VP4 and VP7 from all the four human serotypes would be ideal. Inclusion of one or both types of the inner capsid protein, VP6, might potentiate the immunogenicity as well as stability of the recombinant vaccine.

We have cloned, characterized and determined the nucleotide sequence of genes encoding VP4, VP6 and VP7 from two human serotypes and are being expressed in bacteria towards the goal of producing reagents for a recombinant rotavirus vaccine.

Reassortant rotavirus vaccine might be more potent and economical compared to recombinant vaccine which involves expression and purification of two antigenic proteins from multiple serotypes.

7. Structure and function of nonstructural proteins

Rotavirus genes 5, 7, 8, 10 and 11 code for nonstructural proteins. The function of nonstructural proteins in regulation of virus replication, transcription, translation and pathogenicity is not clearly understood. Rotaviruses exhibit host range restriction in terms of infection and pathogenicity. The fact that both VP4 and VP7 of I321 are of bovine origin, suggest, for the first time, that host range restriction might be encoded by one of the genes encoding the nonstructural proteins. Comparative study of the function of nonstructural proteins from symptomatic human rotaviruses and I321 would lead to the identification of genes responsible for viral pathogenicity and host range restriction.

Currently, we are also examining Indian bovine rotaviruses to determine the genetic origin of the asymptomatic neonatal rotaviruses.

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