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Evidence for a new rotavirus subgroup in India

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SUMMARY

Monoclonal antibodies specific for rotavirus subgroup 1 (SG1) and subgroup 2 (SG2) were used to analyse by enzyme immunoassay (EIA) the subgroups of human rotavirus isolates obtained from three different parts of India during the period September 1985 to July 1987. We identified one isolate which failed to react with either SG1 or SG2 specific monoclonal antibodies, although it reacted well with a monoclonal antibody specific for group A rotaviruses. This finding suggests that it belongs to a new rotavirus subgroup. Further, another isolate was found to belong to SG1 although it had a 'long' electropherotype.

INTRODUCTION

Rotaviruses are the most common causative agent associated with acute gastroenteritis in infants and young children in developing as well as developed countries (Kapikian *et al.* 1976; Holmes, 1979). Detailed serological characterization of rotavirus isolates from different parts of the world has become an essential part of preparing preventive measures. Serotyping of rotaviruses directly from stool specimens is still difficult and, although serotype specific monoclonal antibodies are being used for serotyping (Beards, 1987; Coulson *et al.* 1987; Taniguchi *et al.* 1987), they are not yet available to all laboratories. The alternative methods of typing strains require more advanced biochemical techniques such as the preparation of gene probes (Midthun *et al.* 1987). Isolation of rotaviruses in cell culture and subsequent serological analysis is difficult and often impossible.

Five immunologically different groups of rotaviruses have been identified so far (Pedley *et al.* 1986) but viruses of group A are found most commonly. The group-specific antigen of group A rotaviruses is a major inner capsid protein of 42000 Da (VP6) and, in human isolates, two subgroups have been described, SG1 and SG2 (Kapikian *et al.* 1981; Greenberg *et al.* 1983). The accuracy with which isolates can be subgrouped has been increased greatly with the development of monoclonal antibodies specific for the two subgroups (Greenberg *et al.* 1983; Taniguchi *et al.* 1984).

Rotaviruses can also be characterized by the electrophoretic migration of their 11 double-stranded RNA segments and this technique has potential as an epidemiological tool (Estes, Graham & Dimitrov, 1984). Subgroup analysis of human rotaviruses from diverse geographic areas (Kalica *et al.* 1981; Kutsuzawa

et al. 1982; Greenberg *et al.* 1983; White *et al.* 1984) have shown that SG2 rotaviruses have a 'long' electrophoretic migration pattern of their 10th and 11th genomic segments while SG1 rotaviruses show a 'short' electrophoretic migration.

In the present study we have identified rotaviruses by polyacrylamide gel electrophoresis (PAGE) by investigating 309 stool samples of hospitalized children with diarrhoea from three geographically distinct locations of India: Calcutta (220 samples), Manipur (81 samples) and New Delhi (8 samples positive by EIA for rotavirus). Their subgroup identities have been analysed with SG1- and SG2-specific monoclonal antibodies originally developed by Greenberg *et al.* (1983). The correlation between subgroup and electropherotype of these isolates was also examined.

MATERIALS AND METHODS

Reference human rotaviruses used in this study were Wa (SG2, serotype 1), DS-1r (a recombinant between the UK strain of bovine rotavirus and the DS-1 strain of human origin; SG1, serotype 2), Hochi (SG2, serotype 4) and 69M (SG1, serotype 5; Matsuno *et al.* 1985). The simian rotavirus SA11 (SG1, serotype 3) was obtained from Dr M. K. Estes of Baylor College of Medicine, Houston, Texas. All other viruses were received from Dr T. H. Flewett, Regional Virus Laboratory, East Birmingham Hospital, UK. The viruses were grown in the monkey kidney cell line MA 104 (also supplied by Dr Flewett) as described by Graham & Estes (1980). They were plaque-purified and their genomic RNA pattern in PAGE were confirmed. Rotavirus RNA from stool samples was obtained by the phenol-chloroform extraction procedure described by Herring *et al.* (1982). Cell culture-grown rotaviruses were first pelleted by ultracentrifugation at 100000 g for 1·5 h and the RNA extracted by the same phenol-chloroform procedure. Viral RNA segments were separated on 7·5% polyacrylamide slab gels with a 4% stacking gel, using the discontinuous buffer system of Laemmli (1970) but without SDS. The gels were run at a constant 10 mA current for 12 h and silver stained as described by Herring *et al.* (1982).

The monoclonal antibodies specific for SG1 rotaviruses (AF 255/60), SG2 (AF 631/9) and for group A rotaviruses (AF A3M4) used in study were obtained from Dr Flewett in the form of ascitic fluids. Enzyme immunoassays for subgrouping were carried out in 96-well microtiter plates (Immunoplate I, Nunc, Denmark), following the method of Follett *et al.* (1984) with some modifications. A rotavirus group A-specific antiserum against incomplete SA 11 particles was prepared in our laboratory and was used at a dilution of 1/6000. In comparison, the monoclonal antibodies were used at a dilution of 1/10000. The optical density (OD) values were read in a Bio-Rad model 2550 EIA reader at a wavelength of 405 nm and a ratio of 2·0 or higher between the OD values observed with the SG1 monoclonal antibody over the SG2 monoclonal antibody was used to classify a rotavirus at SG1. Similarly, specimens with an SG2/SG1 OD ratio of 2·0 or higher were classified as SG2. Specimens with SG1/SG2 ratio or SG2/SG1 ratio lower than 2·0 could not be classified. Those samples which could not be classified into a subgroup in the first EIA or in a repeat test were clarified further. A 10% faecal suspension was again prepared, mixed with an equal volume of fluorocarbon (1, 1,2-trichlorotrifluoroethane, E. Merck, Darmstadt, FRG) and mixed thoroughly in a

Table 1. *Subgrouping results by EIA of the two unusual isolates*

	Average OD values (at 405 nm) with monoclonal antibodies specific for			Classified subgroup by EIA	RNA pattern*
	Subgroup 1 (AF 255/60)	Subgroup 2 (AF 631/9)	Group A rotavirus (AF A3M4)		
Samples					
BCH 96	0·026 ± 0·009†	0·027 ± 0·007	0·749 ± 0·119	?	L
D 610	0·805 ± 0·080	0·030 ± 0·021	0·945 ± 0·107	1	L
Controls					
Wa	0·012 ± 0·009	0·594 ± 0·027	1·343 ± 0·046	2	L
DS-1r	1·384 ± 0·017	0·007 ± 0·005	1·611 ± 0·083	1	S
SA 11	1·269 ± 0·148	0·007 ± 0·005	1·588 ± 0·181	1	L
Hochi	0·035 ± 0·004	1·259 ± 0·037	1·496 ± 0·095	2	L
69 M	1·422 ± 0·112	0·041 ± 0·012	1·701 ± 0·147	1	Super short

* L, long; S, short.
† Arithmetic mean ± S.D. for $n = 3$ determinations.

vortex mixer for 1 min. Following centrifugation at 5000 g for 10 min, the aqueous phase was collected from the top and used in the subgrouping EIA. Cell culture-grown strains of the reference rotaviruses were included in all tests as controls.

RESULTS

In the 22 months of the study (from September 1985 to July 1987) 47 (21·4%) out of 220 stools from Calcutta were positive for rotaviruses. This period included two winter seasons and, as in other studies (Kapikian *et al.* 1976; Saha *et al.* 1984), the prevalence of rotavirus infection was higher in these colder months. Sixteen (34·0%) of the isolates from Calcutta had a short RNA pattern by PAGE. The specimens from Manipur were collected between February and April 1986 and 33 (40·7%) out of 81 isolates were positive for rotaviruses. Six (18·2%) of these had a short pattern. In addition, a mixed infection containing both a short and a long electropherotypes was found in one sample from Manipur. Out of 8 positive isolates from New Delhi, 3 had a short pattern.

From these 88 rotavirus-positive cases, sufficient stool was available from 81 samples for subgroup analysis. Fifty samples were classified as SG2 and 24 as SG1. One sample (the one with a mixed infection) reacted with both SG1 and SG2 specific monoclonal antibodies. The subgroup could not be determined for the other six isolates in the initial or the repeat EIA. Their reaction to the group A-specific monoclonal antibody were measurable but their reactions with SG1 and SG2 specific monoclonal antibodies were very weak and inconsistent. Following fluorocarbon extraction, their reactions to the three monoclonal antibodies became consistent and five of the above isolates were successfully subgrouped (all were SG2). However, one isolate (BCH 96) repeatedly failed to react to SG1- or SG2-specific monoclonal antibody. All tests were repeated at least three times and an average of the OD values was taken for final analysis. The EIA results on this isolate are presented in Table 1.

In this study there was a good correlation between the electrophoretic

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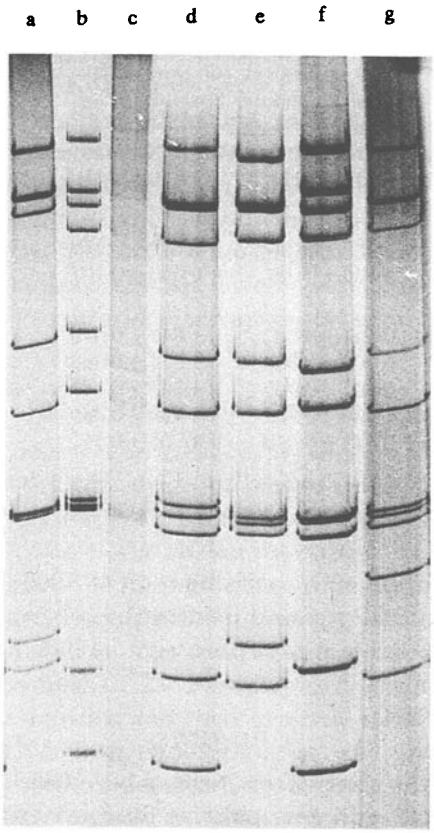

Fig. 1. Electrophoretic migration pattern of rotavirus reference strains and of isolates of this study. Lane a, DS-1r; b, SA 11; c, BCH 96 (unclassified subgroup); d, D 610 (SG1); e and f are typical short and long-pattern human rotaviruses with SG1 and SG2 specificities, respectively, isolated in this study; g, 69 M.

Table 2. *Subgroup analysis and electropherotypes of the rotavirus isolates from three geographically distinct locations of India*

Source	Number of samples tested	Subgroup 1*	Subgroup 2	Unclassified subgroup
Calcutta	42	14(14S)	27(27L)	1(L)
Manipur†	32	6(6S)	25(25L)	—
New Delhi	7	4(3S, 1L)	3(3L)	—
Total	81	24(23S, 1L)	55(55L)	1(L)

* L, long RNA pattern, S, short RNA pattern

† One sample from Manipur reacted to both SG1 and SG2 monoclonal antibodies and had a mixed electropherotype; it has not been considered for subgrouping.

migration of the 10th and 11th gene segments and subgroup identity in 79 out of the 81 rotavirus positive samples. All 24 isolates with a short RNA pattern were subgrouped as SG1; while 55 out of 57 strains showing a long RNA pattern were SG2, one was SG1 and one remained unclassified. The EIA results with the unclassified strain (BCH 96, Fig. 1, lane c) and the one which showed a long RNA

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pattern and SG1 characteristics (D 610, Fig. 1), lane d) are presented in Table 1. The distribution of SG1 and SG2 in the different geographic locations and their electrophoretic migration patterns are shown in Table 2.

DISCUSSION

Until the recent study by Nakagomi *et al.* (1985), all studies (Greenberg *et al.* 1983; White *et al.* 1984) have shown that all long pattern human rotaviruses were SG2 and all short patterns were SG1. On the other hand, rotaviruses from animal sources usually show a long RNA pattern with SG1 characteristics (Beards, 1982; Greenberg *et al.* 1983). However, two independent groups of workers (Garbarg-Chenon, Bricout & Nicolas, 1986; Urasawa, Urasawa & Taniguchi, 1986) have reported the generation *in vitro* of reassortant human rotavirus strains which have either a short RNA pattern associated with SG2 specificity or a long pattern with SG1 characteristics. Apart from these laboratory-generated strains, only one human isolate (Nakagomi *et al.* 1985) has so far been isolated which has a long pattern with SG1 characteristics. This strain has been claimed (Nakagomi *et al.* 1987) to be an animal rotavirus that has infected man. We have now found another such strain, D 610, with a long RNA pattern and SG1 characteristics. Samples for RNA-PAGE and subgrouping EIA were prepared fresh in all repeat tests and care was taken to label the samples correctly. We have not yet tested the genetic relatedness of this strain to animal rotaviruses. The reverse (a strain with short RNA pattern and SG2 characteristics) has also been found in nature but again only once (Steele & Alexander, 1988). The group/subgroup antigens of rotaviruses are coded by their 6th genome segment, whereas the 'long' and 'short' electropherotype classification depends on the mobilities of the 10th and 11th genome segments. Therefore, it is not surprising that the subgroup specificities and short/long RNA patterns should reassert independently. However, only few human strains have been isolated so far which do not have the usual pairing of either SG1 with a short RNA pattern or SG2 with a long RNA pattern.

In our study fluorocarbon extraction of the stool samples improved the consistency of their reactions with the monoclonal antibodies. However, 5 of the 6 isolates whose subgroup could be determined only after fluorocarbon extraction, showed very weak reactions to the subgroup-specific monoclonal antibodies. Though SG2/SG1 OD value ratios of more than 2 were obtained, their reactions against SG2 specific monoclonal antibodies were never more than 0·2 OD unit (data not shown). The sixth isolate, BCH 96, did not show any measurable reaction to either of the subgroup specific monoclonal antibodies. Since this strain reacted well with the group A rotavirus-specific monoclonal antibody (Table 1), it is unlikely that the subgroup-specific monoclonal antibodies could not react with virus antigen. It is also apparent from the low values for the standard deviation, that the EIA tests were consistent. This strain may therefore belong to a subgroup different from either SG1 or SG2. The strain showed a long RNA pattern and it is interesting to note that two other papers (Lambert *et al.* 1983; Tüfvesson, 1983) have reported rotavirus strains which appear to belong to neither SG1 nor SG2. However, in both these reports polyclonal antisera were used to assign subgroups. Tüfvesson (1983) used crossed-immunoelectrophoresis and EIA, whereas Lambert

et al. (1983) used a complement fixation (CF) test to detect subgroups of human rotaviruses. The classification of rotaviruses using polyclonal antisera may not necessarily reflect antigenic differences only in the inner capsid protein VP6 which has been identified as the subgrouping component of rotavirus. Nevertheless Lambert *et al.* (1984) found great similarity between their CF results and the typing results of Thouless, Beards & Flewett (1982) either by neutralization or by EIA, and also to the plaque reduction assay results of Wyatt *et al.* (1982). Though Lambert *et al.* (1984) were able to raise a monoclonal antibody specific for their SG3 rotaviruses, the virion protein specificity of that antibody is not known. In contrast, virion protein specificities of the SG1 and SG2 specific monoclonal antibodies used in this study are well established (Greenberg *et al.* 1983). Thus failure to subgroup one of our isolates with the monoclonal antibodies AF 255/60 or AF 631/9, indicate the existence of a human rotavirus strain with a variation in its major inner capsid protein VP6. We are in the process of cultivating this strain to facilitate further characterization of its protein.

Recently it has been shown by Hoshino *et al.* (1987) that a group A equine rotavirus Fl-14 possesses both SG1 and SG2 specific epitopes on its VP6. They were able to develop both SG1 and SG2 specific antibody producing monoclones from this Fl-14 strain. Further, four non-human rotavirus isolates of different serotypes and four different species, H-2 (horse), Ch2 (chicken), Ty1 (turkey) and SW (mouse), were identified in this study which belonged to neither SG1 nor SG2, although they all were group A rotaviruses. Whether these peculiarities of the subgroup antigens are due to genetic recombination of the 6th genome segment *in vivo* or to evolutionary changes have yet to be established. However, these findings suggest that variations are possible and presumably our non-SG1/SG2 human group A rotavirus strain (BCH 96) originated in a similar way.

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