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Direct Isolation in Cell Culture of Human Rotaviruses and Their Characterization into Four Serotypes

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Of 73 rotavirus-positive fecal specimens tested, 39 yielded a human rotavirus that could be cultivated serially in MA104 or primary African green monkey kidney cells or both; 18 were serotyped. Four distinct serotypes were identified by plaque reduction or tube neutralization assay or both, and three of these serotypes were the same as those established previously by plaque reduction, using human rotaviruses cultivated by genetic reassortment with a cultivable bovine rotavirus. Ten human rotavirus strains received from Japan were found to be similar, if not identical, to our candidate prototype strains representing these four human rotavirus serotypes.

Previous inability to cultivate human rotaviruses hindered the serotypic characterization of these viruses by neutralization assay. The fluorescent-focus neutralization assay was used successfully, however, since the virus was not required to undergo efficient replication or to produce a cytopathic effect (CPE) (3, 23). Subsequently, after serial passage in gnotobiotic piglets, a single strain of human rotavirus, Wa, was successfully propagated in primary African green monkey kidney (AGMK) cells (28). This strain was characterized by both plaque reduction neutralization assay and fluorescent-focus neutralization assay and was found to be distinct from selected animal rotavirus strains (28, 29). More recently, Japanese investigators demonstrated successful isolation of multiple human rotavirus strains in roller tubes of MA104 cell cultures, using trypsin (21, 25). Successful cultivation of human rotaviruses has also been reported in primary cynomolgus monkey kidney cells (10). These newly cultivated strains have been characterized by neutralization of plaques or CPE and were found to represent three to five distinct serotypes (20, 24). An enzyme immunoassay for distinguishing three serotypes of human rotavirus from clinical specimens has recently been described, in which antisera absorbed with heterologous rotaviruses were used (22).

Human rotaviruses were also cultivated or "rescued" by the technique of genetic reassortment, in which a temperature-sensitive mutant of bovine rotavirus was reassorted with a noncultivable human rotavirus under the selective pressure of temperature and high-titered hyperimmune antiserum, so that the resultant viruses exhibited the growth characteristics of the bovine parent and the neutralization phenotype of the human parent (7, 9). Human rotaviruses rescued in this manner and characterized by plaque reduction neutralization assay fell into three distinct serotypes (26). Serotypes could be correctly predicted by using a single dilution of high-titered hyperimmune antiserum in a simplified CPE assay (9). The use of the genetic reassortment technique also permitted the assignment of function to selected genes (6, 13, 14). Of particular interest is the observation that, depending on the rotavirus strain, gene 8 or 9 codes for the primary neutralization determinant (6, 14). Thus, in this description of serotypic characterization, it is likely that antibody to the product of gene 8 or 9 is primarily responsible for neutralization, although recently a neutralization function has also been assigned to the products of genes 4, 6, and 10 (1, 8a, 13, 16).

In this study, 18 of 39 human rotavirus strains, which were cultivated directly in cell culture, were classified into four distinct serotypes by the use of plaque reduction assay or tube neutralization read by enzyme immunoassay or both. These strains were further compared with other human rotavirus strains isolated in Japan (17, 20, 24).

MATERIALS AND METHODS

Cell cultures. MA104 cell cultures were obtained originally from E. H. Bohl, Ohio Agricultural Research and Development Center, Wooster, and were

prepared either at MA Bioproducts, Walkersville, Md., or in our laboratory. An effort was made not to exceed passage 72. Cultures of primary AGMK were purchased from MA Bioproducts.

Viruses. Rotaviruses for primary isolation were in the form of diarrheal or normal feces, either as approximate 10% suspensions or as rectal swab fluids. Seventy-three such fecal samples containing rotavirus were kindly supplied by the following investigators: H. W. Kim and colleagues, Children's Hospital National Medical Center, Washington, D.C.; I. Perez and colleagues, Hospital de Niños, Caracas, Venezuela; J. Banatvala and colleagues, St. Thomas Hospital, London, England; R. Bishop and colleagues, University of Melbourne, Melbourne, Australia; J. G. Kapsenberg, Rijkinstituut Voor de Volksgezondheid, Bilthoven, The Netherlands; R. Black and colleagues, University of Maryland, Baltimore, Md.; R. Yolken, Johns Hopkins University, Baltimore, Md.; and O. Sobeslavsky, who was coordinating a collaborative study of diarrheal diseases for the World Health Organization. Selected rotavirus strains were previously passaged one to three times in gnotobiotic calves or piglets before inoculation into cell cultures as a part of collaborative studies with C. A. Mebus and A. Torres-Medina at the University of Nebraska, Lincoln, and with E. H. Bohl, L. J. Saif, and K. W. Theil at the Ohio Agricultural Research and Development Center, Wooster. The isolation and characterization of human/bovine rotavirus reassortants were previously described (9). Human rotavirus strains were received from Japan from S. Urasawa, T. Konno, Y. Inaba, and their colleagues in the form of cell culture suspensions of cultivable viruses.

Cultivation method. The method of Sato et al. was used for cultivation of human rotaviruses with only minor modifications (21). The first nine viruses inoculated into cell culture were partially purified as described before (21), but subsequently 10% suspensions or rectal swab fluids were used directly for inoculation. Before inoculation, virus suspensions were treated with 10 μg of trypsin (type IX; Sigma Chemical Co., St. Louis, Mo.) per ml for 1 h at 37°C. After an adsorption period of 1 h at 37°C, tubes were washed once and fed with Eagle minimum essential medium containing 0.5 μg of trypsin per ml. DEAE-dextran was added to the medium at a concentration of 100 μg/ml for use on MA104 cells (18), but was omitted for use on AGMK cells because of toxicity for the cells. Eagle minimum essential medium was prepared according to the description of Sato et al., except for the omission of sodium glutamate (21). Cells were incubated for 3 to 10 days on a roller apparatus at 37°C and were observed for CPE. After that time, cells were frozen and thawed once and then tested by indirect enzyme immunoassay for viral antigens (15).

Hyperimmune antisera. Antisera were prepared as previously described (7, 9, 26). Hyperimmune antisera against selected Japanese rotavirus strains were kindly supplied by S. Urasawa and T. Konno.

Plaque reduction assay. Rotaviruses were plaqued in six-well dishes of MA104 cells as previously described (27). For neutralization assay, the hyperimmune antisera were inactivated at 56°C for 30 min. Fourfold dilutions were then prepared and mixed with a dilution of trypsin-treated virus calculated to yield approximately 30 PFU per well (27). Antibody titers were estimated based on 60% reduction of plaque count. Viruses that exhibited a reciprocal 20-fold or greater difference in serum antibody titer were considered distinct serotypes (26).

Modified tube neutralization assay read by enzyme immunoassay. Approximately 10 to 100 50% tissue culture infective doses of trypsin-treated virus were incubated with 10-fold dilutions of inactivated hyperimmune antisera before inoculation into one tube each of MA104 roller tube cultures under the same conditions described above. After 2 or more days of incubation, the tubes were harvested and examined by indirect enzyme immunoassay for rotavirus antigens (15) (Fig. 1). The antibody titer was considered to be the highest dilution of antiserum which completely neutralized the virus as measured by indirect enzyme immunoassay. A 20-fold reciprocal difference in antibody titers was considered the basis for differentiating serotypes (26).

Subgrouping analysis. Rotavirus fecal suspensions and cell culture isolates were analyzed for subgroup antigen by the use of monoclonal antibodies as previously described (8), except that indirect enzyme immunoassay was used instead of radioimmunoassay in some cases. Goat anti-mouse immunoglobulin G conjugated with alkaline phosphatase (Kirkegaard and Perry, Inc., Gaithersburg, Md.) was used in the reaction, and human rotaviruses DS-1 and Wa, subgroup 1 and 2 viruses, respectively, were used as positive controls.

RNA analysis by polyacrylamide gel electrophoresis. RNA was extracted from rotavirus preparations and examined on polyacrylamide gels as previously described (14).

RESULTS

Cultivation of rotaviruses. Thirty-five strains of human rotavirus were successfully cultivated from 73 rotavirus-positive fecal specimens, using MA104 cell cultures. These strains were passaged serially 5 to 14 times. Eighteen of the 35 fecal samples which yielded rotavirus on inoculation in MA104 cells were also inoculated into primary AGMK cells, and 16 of the 18 grew. Five strains which did not grow in MA104 cells were inoculated into AGMK cells, and four grew. Strains were grown in primary AGMK cells for 3 to 14 passages. Thus, a total of 39 human rotavirus strains were cultivated in cell culture. Growth of rotavirus was determined by indirect enzyme immunoassay, since CPE was variable. Most strains grew well by passage 2 or 3, and cell culture fluids were strongly positive by enzyme immunoassay. Five (14%) of the 35 strains passaged in MA104 cells, however, were not positive in enzyme immunoassay until passage 4 to 6. These same five strains were later inoculated into primary AGMK cells, and four grew during passage 1 and during five subsequent serial passages; one strain grew during two serial passages but failed to grow thereafter. Most strains which could not be serially propagated in cell culture were negative in enzyme

TABLE 1. Serotype and growth characteristics of 19 human rotaviruses isolated directly in cell culture[a]

Strain designation	Country of origin	Yr	Plaques produced	Subgroup no.	Serotype no.	Titer against given serotype[b] determined by: 60% plaque reduction 1	60% plaque reduction 2	60% plaque reduction 3	60% plaque reduction 4	Tube neutralization 1	Tube neutralization 2	Tube neutralization 3	Tube neutralization 4
Wa(G187)[c]	USA	1974	Yes	2	1	≥81,920	<80	<80	<80	≥10,000	<100	<100	—[d]
75-80, D(G25)	USA	1974	Yes	2	1	≥81,920	123	<80	—	≥10,000	<100	<100	<100
Dt(G31)	USA	1974	Yes	2	1	—	—	—	—	1,000	<100	<100	<100
Mo(G171)	USA	1974	Yes	2	1	≥81,920	335	106	—	≥10,000	<100	<100	—
O(G179)	USA	1974	Yes	2	1	≥81,920	231	<80	—	≥10,000	<100	<100	—
77-26, L(G215)	USA	1975	Yes	2	1	≥81,920	<80	<80	—	—	—	—	—
DS-1(G621)	USA	1976	Yes	1	2	248	22,953	<80	<80	<100	≥10,000	<100	<100
HN90II	Venezuela	1981	±	1	2	—	—	—	—	<100	1,000	<100	<100
HN126	Venezuela	1981	Yes	1	2	86	34,149	<80	—	<100	≥10,000	<100	—
HN144	Venezuela	1981	No	1	2	—	—	—	—	<100	1,000	<100	<100
P(G11)	USA	1974	Yes	2	3	646	<80	16,516	—	100	<100	≥10,000	—
263, M(G529)	USA	1976	Yes	2	3	—	—	—	—	<100	<100	≥10,000	<100
WALK 57/14	Hong Kong	1977	No	2	3	—	—	—	—	<100	<100	1,000	—
Pz[e]	Australia	1975	No	2	3	—	—	—	—	100	<100	≥10,000	100
Rv[e]	Australia	1976	No	2	3	—	—	—	—	<100	<100	1,000	<100
ST no. 8	England	1976	±	2	3	285	137	6,985	<80	—	—	—	—
ST no. 4[e]	England	1975	No	2	4	<80[f]	<80[f]	<80[f]	7,441[f]	<100	<100	<100	1,000
ST no. 3[e]	England	1975	±	2	4	162[f]	<80[f]	<80[f]	11,971[f]	100	<100	<100	≥10,000
ST no. 6[e]	England	1976	No	2	4	—	—	—	—	100	100	<100	≥10,000

[a] Twenty-one additional strains were grown but not yet characterized for serotype: Mo(G287); Ti; Ca; Gr; McN; YIP no. 43 (no. 15); 77-27, DS-2(G965); 77-11, G(G213); 77-25, W(G999); 77-28, Fh(G57); 80-3234; 80-3740; 80-4993; 80-7173; HN219; M22; M37; ST no. 1; ST no. 2; ST no. 5; and ST no. 7.

[b] Titers expressed as reciprocal values, using high-titered specific antiserum against indicated serotype (1, Wa; 2, DS-1; 3, WALK 57/14 reassortant, or rhesus rotavirus MMU18006; and 4, ST no. 4).

[c] Reference 28.

[d] —, Not tested.

[e] Specimens derived from asymptomatic newborns.

[f] Since direct isolate does not plaque adequately, human/bovine reassortant was used as antigen in plaque reduction assay (9).

immunoassay by passage 2. Twenty-three of the 73 fecal samples were tested in the form of rectal swabs, and none of these specimens yielded a cultivable rotavirus. The 39 isolates were recovered from 50 fecal suspensions tested in the same manner as the rectal swabs, thus yielding an isolation rate of 78% from such specimens.

Twenty-six of 39 cultivated rotavirus strains were derived from fecal specimens obtained from infants and young children who had an acute diarrheal episode for which medical attention was sought. The remaining 13 cultivated strains were derived from fecal samples obtained from 20 neonates who were asymptomatic (4, 19).

In addition, 10 tissue culture-adapted human rotavirus strains (K8, KU, S2, YO, KUN, MO, Ito, Nemoto, Hosokawa, and Hochi) received from Japan were passaged further in MA104 cells (17, 20, 24).

Plaquing of rotavirus isolates. Thirty-five isolates were studied for plaque formation in MA104 cells. Only 11 (31%) produced easily recognized plaques, whereas 12 (34%) produced very small and faint plaques. Twelve isolates (34%) did not produce any plaques. Thus, not all strains could be tested for serotype by plaque reduction assay as previously described for human/bovine reassortants, which usually produce clear plaques at a titer of $>10^6$ PFU/ml. Titers of isolates recovered directly in MA104 cells ranged from 10^2 to 10^6 PFU/ml, and CPE in the roller tube cultures was variable.

Determination of serotype. Twenty-nine of the 39 strains, including Wa as a reference strain, which were cultivated directly in cell culture were examined by plaque reduction or modified tube neutralization assay or both with hyperimmune antisera to the three previously described human rotavirus serotypes (Table 1). Serotype was established for 18 of the 29; results with the remaining 11 were inconclusive. Eight of the 18 were classified by plaque reduction assay as belonging to one of the three distinct serotypes described previously (Table 1) (26). Four additional human rotaviruses, designated St. Thomas (ST) no. 1, 2, 3, and 4, in the form of human/bovine reassortants (9), were also examined by plaque reduction assay and found to be distinct from these three serotypes (only data for ST no. 3 and 4 are shown in Table 1). With the use of hyperimmune serum developed against the ST no. 4 strain isolated directly in MA104 cells, it was then established that this strain was in fact a fourth serotype (Table 2). Although these four strains were also available as direct isolates, they did not produce distinct enough plaques to permit characterization by the plaque reduction assay.

A modified roller tube neutralization assay was used to study 22 of the 39 direct human rotavirus isolates, including the previously cultivated Wa strain. Fourteen of the 22 could be assigned to one of the three serotypes (Wa, DS-1, and M) previously reported (Table 1). The tube neutralization assay also clearly established the ST no. 3, 4, and 6 isolates as representing a serotype distinct from the three previously es-

TABLE 2. Composite of two plaque reduction assays which demonstrate the presence of a fourth rotavirus serotype

Rotavirus	Reciprocal of titer against hyperimmune antiserum to given serotype				
	Wa	DS-1[a]	WALK 57/14[a]	Rhesus (MMU18006)	ST no. 4
Wa	≥81,920[b,c]	—[d]	—	—	<80[b] 84[c]
DS-1	—	≥81,920[c]	—	—	<80[c]
DS-1[a]	—	≥81,920[b]	—	—	<80[b]
WALK 57/14[a]	—	—	75,035[b]	—	<80[b]
Rhesus (MMU18006)	—	—	—	≥81,920[c]	<80[c]
ST no. 4[a]	<80[b,c]	2,229[b]	<80[b]	<80[c]	7,322[b] 3,895[c]

[a] Human/bovine reassortant (9).
[b] Values were obtained in test no. 1; antiserum to DS-1 is different from that used in tests shown in Table 1. Homologous values are underlined.
[c] Values were obtained in test no. 2. Homologous values are underlined.
[d] —, Not tested.

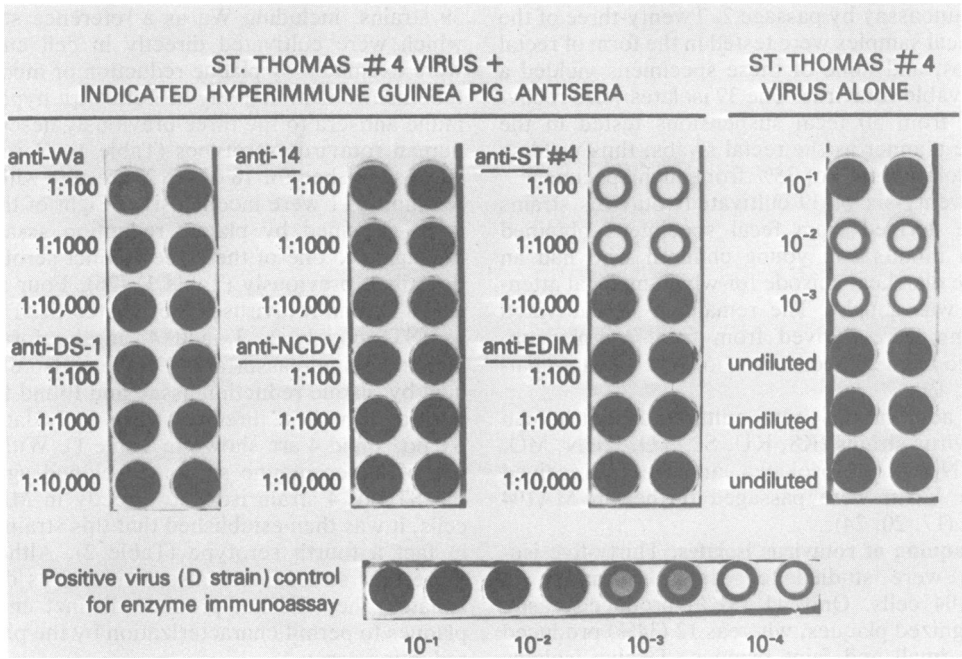

FIG. 1. Serotypic characterizaton of human rotavirus (ST no. 4) by neutralization in MA104 tube culture with viral replication detected by enzyme immunoassay: microtiter plate wells in which tube harvests from a modified neutralization test were assayed by indirect enzyme immunoassay. Hyperimmune guinea pig antisera were diluted 1:100, 1:1,000, and 1:10,000 and mixed with a constant amount of ST, no. 4 virus before inoculation of tube cultures. Back titrations of virus (10^{-1}, 10^{-2}, and 10^{-3}) were included in the test. In this test, human rotavirus strain ST no. 4 was neutralized only by the anti-ST no. 4 antiserum to a dilution of 1:1,000 and not by antisera against Wa, DS-1, WALK 57/14 (indicated by 14), Nebraska calf diarrhea virus (NCDV), or epizootic diarrhea of infant mice virus (EDIM). Microtiter plate variation was controlled by the titration of human rotavirus, strain D, in 10-fold dilutions. A Kodak Wratten no. 49 (blue) filter was used to photograph the microtiter plate to enhance the contrast and density for black and white reproduction.

tablished serotypes (Table 1; Fig. 1). Seven direct isolates were studied by both plaque reduction assay and tube neutralization assay, and both tests yielded the same results in each case. The assignment of strains to a serotype was clear-cut, with reciprocal 20-fold antibody differences being demonstrated in each instance except for strain Pz, which had a low-level one-way relationship with ST no. 4.

RNA analysis. Ten of the newly isolated strains were examined by RNA polyacrylamide gel electrophoresis, and 8 of the 10 were serotyped. Each appeared to be a single strain based on an appropriate number and pattern of RNA segments.

Comparison with Japanese rotaviruses. Ten human rotavirus strains from Japan were compared by plaque reduction assay with the four serotypes encountered in these studies (Table 3). Hyperimmune antisera against Wa (serotype 1), DS-1 (serotype 2), WALK 57/14 (serotype 3), and ST no. 4 (serotype 4) were utilized. Strains K8 and KU were similar if not identical to strain Wa, whereas strains S2 and KUN were similar if not identical to strain DS-1. Strains YO, MO, Ito, and Nemoto were serotyped as being similar if not identical to strain WALK 57/14, although there was a low level of cross-reactivity of Ito with serotype 4 reference antiserum. Strains Hosokawa and Hochi appeared to be similar to ST no. 4 in this one-way comparison. Two-way comparisons were made only for strains K8, KU, S2, KUN, YO, and MO, and in each case a two-way relationship of identity was further established (data not shown). Thus, each of 10 strains from Japan belonged to one of four serotypes established in our studies. The subgroup of each of these 10 strains was determined, and the results are shown in Table 3.

DISCUSSION

Plaque reduction assay and a modified tube neutralizaton assay were used to define the serotype of a series of newly isolated human rotaviruses, and these strains were compared with other strains previously isolated in Japan (17, 20, 24). Other investigators have used the fluorescent-focus neutralization assay or CPE

TABLE 3. Neutralization specificity of human rotavirus strains from Japan as determined with the use of antisera against strains of U.S. origin

Rotavirus	Investigators (reference)	Subgroup no.	Reciprocal of titer by 60% plaque reduction assay against antiserum from guinea pigs hyperimmunized with given rotavirus			
			Wa (≥81,920)[a]	DS-1 (31,273)[a]	Reassortant WALK 57/14 (≥81,920)[a]	ST no. 4 (7,441)[a]
K8	Urasawa et al. (24)	2	≥81,920	<80	<80	<80
KU	Urasawa et al. (24)	2	≥81,920	<80	<80	<80
S2	Urasawa et al. (24)	1	<80	7,227	<80	<80
KUN	Kutsuzawa et al. (17)	1	150	9,347	<80	<80
YO	Urasawa et al. (24)	2	144	<80	72,043	<80
MO	Kutsuzawa et al. (17)	2	<80	<80	35,967	<80
Ito	Sato et al. (20)	2	1,827	320	20,058	304
Nemoto	Sato et al. (20)	2	552	179	42,870	125
Hosokawa	Sato et al. (20)	2	714	<80	<80	813[b]
Hochi	Sato et al. (20)	2	415	<80	<80	862[b]

[a] Reciprocal of homologous titer from previous assay.
[b] Homologous titer of anti-ST no. 4 serum in same test = 1:3,460.

assay to determine serotype (3, 22). All of these assays measure antibody which neutralizes rotavirus and thus characterize the antigenic determinant(s) of the rotavirus which most likely is involved in protective immunity (5, 30).

In this study, we identified four serotypes of human rotavirus isolated from specimens obtained from Australia, England, Hong Kong, the United States, and Venezuela. Ten human rotaviruses isolated previously in Japan were also tested and found to belong to one of the four serotypes. Our findings are in agreement with those of Urasawa et al., which established the existence of three distinct serotypes among strains KU, K8, YO, and S2 (24). The four Japanese strains described by Sato et al. were reported to represent four different serotypes, since at least an eightfold difference between homologous and heterologous antibody titers was observed among these four strains (20). Each strain was also reported to be distinct from strain Wa (20), and our findings confirm that observation. We require a 20-fold difference in homologous and heterologous antibody titers to establish distinct serotypes and tentatively classify these same four strains as belonging to two serotypes, 3 and 4, using our reference antisera. A new fourth serotype emerged from our studies when a series of neonatal specimens from England was examined; the identification in this study of a similar fourth serotype from Japan must be confirmed by the development of high-titered hyperimmune antisera against the strains and comparisons in a two-way fashion.

The numbering system used in this report is consistent with that used by Thouless and colleagues (Table 4) (2, 22). Of interest, strains belonging to the third of these serotypes have

TABLE 4. Comparison of human rotaviruses by serotype

Investigators (reference)	Country	Designation of rotavirus strains which are similar if not identical by neutralization assays			
		1[a]	2[a]	3[a]	4
Beards et al. (3)	England	I	II	III	
Urasawa et al. (24)	Japan	K8 KU	S2	YO	
Kutsuzawa et al. (17)	Japan		KUN	MO	
Sato et al. (20)	Japan			Ito Nemoto	Hosokawa Hochi
Wyatt et al. (26); this study	USA	Wa	DS-1	P[b]	ST no. 4

[a] Designation originally established and currently used by Thouless and colleagues (22).
[b] Also includes rhesus rotavirus MMU18006 and reassortant rotavirus WALK 57/14.

been recovered from simian, canine, and feline species (11, 26). Further comparisons between animal and human rotavirus strains may lead to additional examples of shared serotypes, as occurs with the reoviruses (12). It was also of interest that 5 of the 13 strains isolated from asymptomatic newborns were either serotype 3 (two cases) or serotype 4 (three cases).

Each of the rotaviruses isolated in these studies was determined to be either subgroup 1 or 2. Subgroup specificity represents a function of the gene 6 product of rotaviruses and is not associated with neutralization specificity in a major way (1, 6, 14, 16). However, determination of subgroup in the serial cultivation of rotaviruses offers a simple way of monitoring for contamination by rotavirus of a different subgroup. In addition, it appears that subgroup 1 viruses of human origin belong to serotype 2.

Determination of serotype is often difficult because of poor growth of direct isolates and their failure to produce discrete plaques. Human rotaviruses cultivated or rescued by the use of genetic reassortment plaque well, usually at a titer of 10^6 PFU/ml. Rescue of human rotaviruses is more sensitive than the method of direct isolation used in this study, since eight rotaviruses which were previously rescued from rectal swab preparations could not be grown directly from the same swab preparation in MA104 or AGMK cells. It is also of interest that rotaviruses grown either directly or by reassortment were of the same serotype. Some other methods for determination of serotype which do not require plaquing depend on the development of CPE, which is often variable. Thus, the use of fluorescence or enzyme immunoassay has been useful to measure the presence of rotavirus antigens in neutralization assays (22, 23). The use of enzyme immunoassay in the current study to measure rotavirus replication in roller tube cultures offers the advantage of a sensitive cultivation system, and yet it is not dependent on the development of CPE. The technique is, however, tedious and time-consuming and requires large numbers of roller tube cultures. An enzyme immunoassay has recently been described which is potentially useful to determine serotype on clinical specimens by the use of absorbed antisera (22). This and other simplified solid-phase systems utilizing serotype-specific monoclonal antibodies, probes which will detect specific genes, e.g., gene 8 or 9, or antibodies directed against specific gene products will hopefully allow the rapid determination of serotype required for larger-scale epidemiological studies.

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