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Three Human Rotavirus Serotypes Demonstrated by Plaque Neutralization of Isolated Strains

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Human rotaviruses were isolated directly from stool specimens of gastroenteritis patients in MA-104 cells in the presence of trypsin. For the plaque assay of the isolated strains, the optimal composition of overlay medium was determined. The antigenicity of the isolated strains was investigated by a plaque neutralization method, using antisera prepared against six strains having different electropherotypes of viral RNA, and three different neutralization serotypes were demonstrated.

Until recently, it had not been possible to propagate human rotaviruses in vitro, except for one strain, type 2 Wa strain, which had been adapted to grow in cell cultures after 11 serial passages in newborn gnotobiotic piglets (10). Last year, however, direct isolation of human rotaviruses from stool specimens of diarrheic patients was achieved by using roller tube cultures of MA-104 cells in the presence of trypsin (8, 9). Findings suggestive of cell-to-cell infection of virus, obtained by fluorescent-antibody techniques, as well as the successful adaptation of virus to a stationary culture, prompted us to try the plaque formation of virus in MA-104 cells. The preliminary data in this experiment have already been reported (9).

So far in our laboratory, human rotaviruses have been isolated in cell cultures from 20 stool specimens of 24 examined. Of these 20 virus isolates, 8 were previously examined for electrophoretic patterns of viral RNA segments by gel electrophoresis according to Laemmli (6), and five different electropherotypes were found (8a). The analysis of viral RNA segments of another six isolates added four new electropherotypes (unpublished data). Of these nine different electropherotypes found to date, only one (from the S2 strain) showed the "short" electropherotype (2, 7) of viral RNA, in which RNA segments 10 and 11 migrated quite slowly (8a), and the other eight showed the "long" electropherotypes of RNA.

In this communication, the conditions of plaque formation of isolated human rotavirus strains were examined, and this is the first report on the antigenicity of isolated strains investigated by a plaque neutralization method.

Human rotaviruses were cultivated in MA-104 cells by inoculating virus pretreated with acetylated trypsin (Sigma Chemical Co.) (final concentration, 10 μg/ml) and by using medium containing 1 μg of acetylated trypsin per ml (9). To find the optimal composition of agar overlay medium, the effects of several medium components on the plaque formation were examined. Tables 1 and 2 show the results. Experiments with overlay media containing three different kinds of agar and various concentrations of acetylated trypsin revealed that the maximum number of plaques was produced in the combination of 0.6% purified agar (Oxoid Ltd.) and 3 μg of acetylated trypsin per ml (Table 1). Further, the addition of DEAE-dextran to the trypsin-containing agar overlay resulted in the production of clearer plaques, though their sizes and numbers remained unchanged (Table 2).

Based on these results, an overlay medium consisting of Eagle minimum essential medium, 0.6% purified agar, 3 μg of acetylated trypsin per ml and 50 μg of DEAE-dextran per ml was

TABLE 1. Effect of agar and trypsin on plaque formation of human rotavirus, K8 strain

Type of agar	Concn of trypsin (μg/ml)	Plaque diam (mm)	Virus titer (PFU × 10^{-2}/0.1 ml)
Bacto (Difco) 1.0%	3	Pinpoint	31
Noble (Difco) 0.8%	3	Pinpoint	39
Purified agar (Oxoid) 0.6%	1	0.5–1.0	120
	2	0.5–1.0	143
	3	0.5–2.0	177
	4	0.5–2.0	71
	5	0.5–2.0	30
0.8%	1–5	Not detected	0

TABLE 2. Effect of DEAE-dextran on plaque formation of human rotavirus, K8 strain, in medium consisting of 0.6% purified agar (Oxoid) and 3 μg of trypsin per ml

Concn of DEAE-dextran (μg/ml)	Plaque diam (mm)	Virus titer (PFU × 10^{-2}/0.1 ml)
0	0.5–2.0	177
50	0.5–2.0 (clear)	180
100	0.5–2.0 (clear)	178

chosen and used in the following experiments. Under this overlay medium, the majority of tissue culture-grown viruses (17 out of 20 isolates tested) formed plaques of 0.5 to 1.5 mm in diameter 5 or 6 days after inoculation. The viruses were purified by three consecutive plaque isolations to obtain virus clones producing large and clear plaques. Cloned K8 strain (Fig 1b) yielded relatively large plaques, though they were still smaller in size than the Wa strain (Fig. 1a), whereas cloned KU and S12 strains produced medium-sized plaques (Fig. 1c and d). In contrast to these, S2 and YO strains (Fig. 1e and f) produced relatively small and blurred plaques, even after plaque isolations.

To investigate the antigenicity of the isolated strains, antisera were prepared against the six representative strains having different electropherotypes (8a) mentioned above. Immunizing viruses were purified and concentrated by polyethylene glycol precipitation, sucrose density gradient centrifugation, and CsCl banding (8a). Fractions containing single- and double-shelled virus particles were pooled, dialyzed against a buffered saline, and injected once intravenously into weanling rabbits which had been confirmed not to contain neutralizing activity against the Wa strain in their preimmunization sera. Anti-

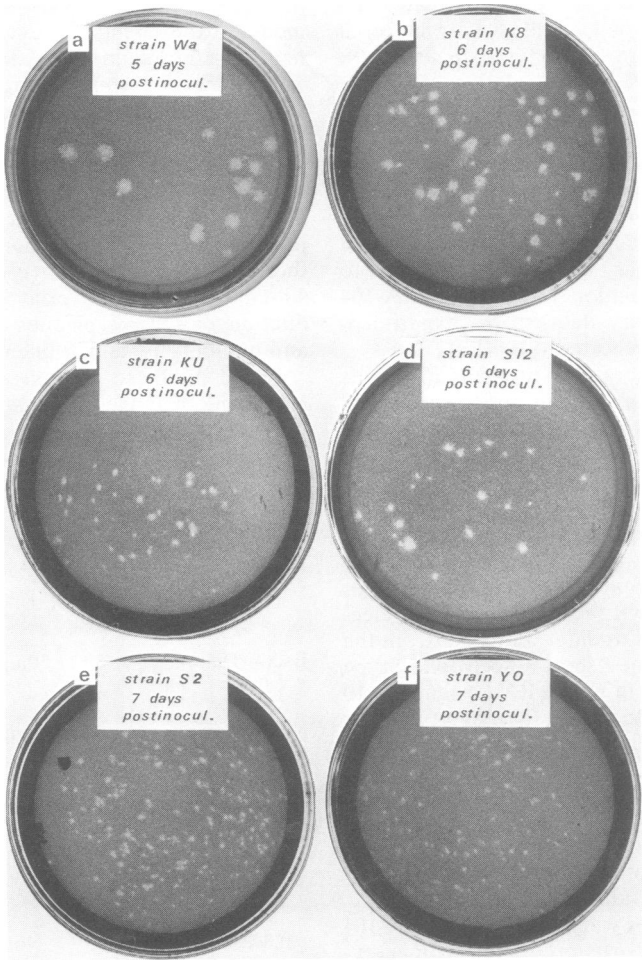

FIG. 1. Plaques of six strains of cell culture-adapted human rotavirus.

TABLE 3. Cross-neutralization of human rotaviruses by the use of a 60% plaque reduction method

Rotavirus	Antibody titer with the following antiserum, pre- and post-immunization:												Serotype[a]
	Anti-S2		Anti-Wa		Anti-KU		Anti-S12		Anti-K8		Anti-YO		
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	
Human													
S2	<32	8,192	<32	<32	<32	<32	<32	<32	<32	<32	<32	32	1
Wa[b]	<32	32	<32	4,096	<32	4,096	<32	32,768	<32	16,384	<32	512	2
Ku	<32	32	<32	1,024	<32	4,096	<32	32,768	<32	8,192	<32	128	2
S12	<32	<32	<32	256	<32	1,024	<32	32,768	<32	8,192	<32	32	2
K8	<32	<32	<32	1,024	<32	4,096	<32	32,768	<32	32,768	<32	64	2
YO	<32	32	<32	<32	<32	<32	64	4,096	32	1,024	64	16,384	3
Calf													
NCDV	<32	<32	<32	<32	<32	256	256	2,048	<32	2,048	<32	1,024	

[a] Tentative.
[b] Kindly supplied by R. G. Wyatt, National Institutes of Health, Bethesda, Md.

sera were obtained 10 to 20 days after immunization. Cloned viruses were employed for both the immunization of animals and neutralizing antibody titration. The antibody titers were determined by a 60% plaque reduction method, using ca. 150 PFU of virus.

The results are shown in Table 3. The three rabbits used to immunize S12, K8, and YO strains had a low level of neutralizing activity against either the YO strain or both the YO strain and the calf rotavirus, NCDV, before immunization. The possibility, therefore, could not be excluded in the present study that this preexisting immunological status of animals influenced the post-immunization titer against these two viruses and consequently obscured the significant antigenic differences. Under these conditions, an eightfold or greater difference between the post-immunization titers measured with the homologous and heterologous strains was regarded to indicate distinct antigenic differences of strains in this study. According to this criterion, S2 and YO strains were found to represent two distinct neutralization antigens. In contrast, the neutralization patterns obtained by the four remaining strains, Wa, KU, S12, and K8 indicated that these strains are mutually related antigenically since antisera against KU, S12, and K8 strains strongly neutralized all four strains, though anti-Wa serum did not significantly neutralize the S12 strain.

On the basis of these results, KU, K8, and S12 strains possessing antigenicity related to the type 2 Wa strain and having the "long" electropherotype of viral RNA (8a) are thought to be classified as serotype 2, whereas the S2 strain serologically unrelated to the Wa strain and characterized by the "short" electropherotype of RNA (8a) may well be classified as serotype 1, considering its analogy to the non-cultivatable DS-1 strain of type 1 human rotavirus (3). In contrast to these, the YO strain, which is antigenically different from all of the other strains, seems to represent serotype 3.

Recently, human rotavirus antigens detected by cell culture neutralization were found to be distinct from those detected by an immune adherence hemagglutination assay (IAHA) or an enzyme-linked immunosorbent assay (ELISA) (3, 5). Further, it was reported that neutralization and IAHA antigens were encoded by different RNA segments, 9 and 6, respectively (4). Based on these results, it was proposed that the term "serotype" be reserved to identify the specificity of antigen reacting with neutralizing antibodies and that the term "subgroup" be used for the specificity detected by specific complement fixation, ELISA, and IAHA (5).

As to the neutralization serotypes of human rotavirus, the presence of three different types was shown very recently, using "non-cultivatable" human rotaviruses that were rescued by coinfection with a cultivatable bovine rotavirus (3a, 9a). Beards et al. (1), on the other hand, reported at least three and possibly five different serotypes determined by a fluorescent focus neutralization method. In this communication, we described the presence of three different serotypes in human rotavirus by a plaque neutralization method, using viruses isolated directly from diarrheal patients and the respective antisera.

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