Plaque Assay of Neonatal Calf Diarrhea Virus and the Neutralizing Antibody in Human Sera

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Neonatal calf diarrhea virus (a bovine rotavirus) formed distinct plaques in monolayers of MA-104 cells, an established macacus rhesus monkey kidney cell line, when diethylaminoethyl dextran and trypsin were included in the overlay medium. By using this plaque assay method, titration of neutralizing antibody to neonatal calf diarrhea virus was made feasible. It was demonstrated that some human sera contained neutralizing antibody to this agent.

In 1973 a reovirus-like agent was discovered by electron microscopy in duodenal biopsies (1) and in fecal specimens (3) from infants with acute gastroenteritis. It was eventually confirmed in this country that morphologically similar agents are responsible for infant gastroenteritis occurring in winter (9). Moreover, in the spring of 1976, when outbreaks of gastroenteritis were noted in primary schools in several districts, similar reovirus-like viruses were detected in fecal extracts from some patients (5). However, no reproducible and practicable methods of growing the agent in cell culture are available at present.

Kapikian et al. (6) reported that neonatal calf diarrhea virus (NCDV), which is morphologically similar to the human infantile gastroenteritis agent, is serologically related to the human virus in complement fixation (CF) tests. NCDV has been adapted to growth in cell cultures (2, 10, 11), and its CF antigen is now widely used for serological studies of human gastroenteritis cases (4, 8). In our experience the cytopathic effect caused by NCDV in cell cultures is not easily recognizable; therefore, reliable plaque assay techniques for NCDV have not yet been described (11).

In the present report we describe (i) a plaque assay technique for NCDV developed by us and (ii) the results of plaque reduction neutralization (NT) tests with serum specimens from diarrhea patients.

MATERIALS AND METHODS

Virus. The Lincoln strain of wild-type NCDV passaged in primary bovine kidney cells was kindly supplied by M. Kono, National Institute of Animal Health, Kodaira, Tokyo. The virus was inoculated into primary cynomolgus monkey kidney cells (PCMK) and incubated at 37°C. When extensive

cytopathic effect was observed, the cells were harvested and then frozen and thawed three times. After low-speed centrifugation ($800 \times g$ for 10 min), the supernatant fluid was stored at -70°C for use as seed virus.

Cell cultures. A continuous cell line, MA-104, which was originally established from macacus rhesus kidney at the Department of Research and Development, Microbiological Associates, Inc., Bethesda, Md., was supplied by D. W. Trent, Vector-Borne Diseases Branch, Center for Disease Control, Fort Collins, Colo. The growth medium was Eagle minimum essential medium in Earle balanced salt solution, supplemented with 10% fetal calf serum and 2.2 mg of NaHCO_3 , 250 U of penicillin, and 50 μg of streptomycin per ml. The cells were dispersed by treatment with a solution of 0.125% trypsin (Difco, 1:250) and 0.02% ethylenediaminetetraacetic acid in cation-free phosphate-buffered saline (PBS) at 37°C for 5 min.

Plaque assay technique. Cell monolayers were prepared in 50-mm plastic dishes (Falcon) by seeding 5×10^5 trypsinized MA-104 cells in 5 ml of growth medium and incubating the cells at 37°C in an atmosphere of 5% (vol/vol) CO_2 for 4 days. After washing with PBS supplemented with 0.2% bovine serum albumin (PBS + BA), serial 10-fold dilutions of virus prepared in PBS + BA were inoculated in 0.1-ml amounts. After 60 min of adsorption at 37°C , each culture received 5 ml of agar overlay medium, which consisted of 0.8% purified agar (Difco) in Eagle minimum essential medium containing 2.2 mg of NaHCO_3 , 100 μg of diethylaminoethyl (DEAE) dextran, and 2 μg of crystalline trypsin (Sigma) per ml. After the agar overlay solidified, the dishes were placed in an inverted position in a CO_2 incubator at 37°C for 3 days. Then an equal amount of a second agar overlay medium, containing 0.8% purified agar in Earle balanced salt solution, 0.003% neutral red (Difco), and 2.2 mg of NaHCO_3 per ml, was added, and plaques were counted the next day.

NT test. Sera were inactivated at 56°C for 30 min. Fourfold dilutions of sera were prepared in PBS + BA. Each dilution was mixed with an equal volume

of the virus suspension containing about 200 plaque-forming units per 0.1 ml and incubated for 60 min at 37°C in a water bath. A 0.1-ml amount of the virus-serum mixture was inoculated onto the cell monolayer. Three plates were used for each dilution. After 60 min of adsorption at 37°C, each dish was washed twice with PBS + BA. Further procedures were exactly the same as described above for the plaque assay technique. The NT antibody titers were expressed as reciprocals of the highest serum dilution causing a 50% reduction in plaque counts.

CF test for NCDV. CF tests were done by the microtiter method, using 5 50% hemolytic units of complement. Antigen was prepared as follows. Infected PCMK cells were freeze-thawed three times. After centrifugation for 30 min at $15,000 \times g$, the supernatant fluid was mixed with 8% polyethylene glycol 6000 and 0.5 M NaCl, kept overnight at 4°C, and then centrifuged at $15,000 \times g$ for 30 min. The pellet was resuspended in PBS to 1/100 of the original volume of fluid, and the CF antigen titer was determined by box titration using hyperimmune guinea pig serum to NCDV.

Preparation of guinea pig antiserum to NCDV. Virus prepared as described above for CF antigen was further centrifuged at $110,000 \times g$ at 4°C for 2.5 h in a Spinco SW41 rotor through a 20 to 50% Metrizamide (Nyegaard) gradient. The visible virus band was collected and, after dialysis against PBS, was mixed with Freund complete adjuvant and injected into footpads of five guinea pigs. Three weeks later, purified virus without adjuvant was injected intraperitoneally. Animals were bled 1 week after the last injection.

Preimmunization serum had a titer of less than 1:4 in CF tests against NCDV and between 1:14 and 1:20 in NT tests. After immunization, the CF antibody titer was 1:4,096 and the NT titer was between 1:40,000 and 1:50,000 with all five guinea pig sera.

Patient serum specimen. Paired serum specimens from 10 gastroenteritis cases in infants and children received during the period from January to April 1976 were examined by CF and NT tests.

RESULTS

Plaque formation by NCDV. Initially, plaque assay of NCDV was attempted in PCMK and various cell lines, including FL, LLC-MK₂, MDCK, MDBK, Vero, PS, and MA-104. Plaques were formed in PCMK, LLC-MK₂, and MDCK cells in the presence of trypsin and DEAE dextran, as described below. However, the results were not reproducible. In contrast, distinct plaques of 2 to 3 mm in diameter were formed consistently in MA-104 cells when both trypsin (2 µg/ml) and DEAE dextran (100 µg/ml) were added to the agar overlay medium. The effect of trypsin and DEAE dextran on NCDV plaque formation was then studied (Table 1 and Fig. 1). Without trypsin and DEAE dextran, no visible plaques appeared even after 6 days of incubation. With an overlay contain-

TABLE 1. Effect of DEAE dextran and trypsin on plaque formation of NCDV

Trypsin (2 µg/ml)	Plaque-forming units/ml	
	+ DEAE dextran (100 µg/ml)	- DEAE dextran
+	1.5×10^7	2.9×10^6
-	4.3×10^5	0

ing DEAE dextran but not trypsin, plaques were small, less than 1.5 mm in diameter, and were often surrounded by a halo. With an overlay containing trypsin but not DEAE dextran, plaques were more numerous and larger, 2 to 3 mm in diameter. Addition of DEAE dextran to the above medium did not result in a further increase in plaque size but did increase plaque numbers by about sixfold. Thus, it was evident that both trypsin and DEAE dextran were required for efficient plaque formation by NCDV, but the contribution of the former was greater. Trypsin at a concentration higher than 3 µg/ml impaired the integrity of the monolayer, resulting in plaques of poorer contrast. Plaques produced with Ionagar no. 2 (Oxoid) or purified agar (Difco or Oxoid) overlays were of equal size, whereas plaques were much smaller under overlays containing Bacto agar (Difco) or Noble agar (Difco).

NT tests with human sera. Five patients shown in Table 2 (no. 1 to 5) were either infants or children under the age of 4 years, whereas the other five cases were school-age children. A significant rise in CF antibody titers was shown in all cases except no. 1, who showed a twofold rise. However, none of infants and younger children with gastroenteritis developed NT antibody. On the other hand, serum specimens from older children contained low or moderate levels of CF and NT antibodies at the onset of the disease, and both the CF and NT antibody levels were further elevated significantly during their convalescence.

DISCUSSION

The plaque assay technique for NCDV was developed under these specific conditions: (i) sensitive MA-104 cells; (ii) an overlay with purified agar or Ionagar no. 2; and (iii) inclusion of both DEAE dextran (100 µg/ml) and trypsin (2 µg/ml) in the agar overlay. Although DEAE dextran and trypsin each enhanced plaque formation, the increase in plaque size was more pronounced with trypsin. DEAE dextran did not increase plaque size beyond that achieved with trypsin alone. However, there was an additive effect of DEAE dextran and trypsin

FIG. 1. NCDV plaques in monolayers of MA-104 cells. (A) Agar containing DEAE dextran (100 $\mu\text{g/ml}$) and trypsin (2 $\mu\text{g/ml}$), 4 days postinfection; (B) agar containing DEAE dextran (100 $\mu\text{g/ml}$) only, 6 days postinfection; (C) agar without DEAE dextran and trypsin, 6 days postinfection.

which resulted in an increase in plaquing efficiency. The exact mechanism of plaque enhancement by these chemicals is not known at present.

Five patients with infantile gastroenteritis showed an antibody titer rise to NCDV in CF

but not in NT tests. On the other hand, a significant increase in antibody was shown by both NT and CF tests for NCDV in five school-age children with similar diseases. Reolike virions have been regularly found by electron microscopy in fecal extracts of infantile gastroen-

TABLE 2. NT and CF antibodies to NCDV in sera from human diarrhea patients

Patient no.	Age (yr)	Sex	Days after onset of disease	NT titer	CF titer
1	1.5	M	7	ND ^a	4
			11	<16	8
2	0.5	M	14	ND	4
			42	<16	16
3	1	M	2	ND	4
			43	<16	16
4	1	M	3	ND	<4
			19	<16	16
5	4	M	2	ND	<4
			6	<16	8
6	8	F	1	200	16
			13	4200	64
7	8	M	1	140	8
			13	250	128
8	8	M	1	300	16
			13	2300	64
9	7	M	1	250	8
			13	4500	64
10	8	F	1	80	<4
			13	960	16

^a ND, Not done.

teritis patients but not always in those from older children (5). Our findings agreed with those of Flewett et al. (4), who detected NT antibody to NCDV by an immunofluorescence technique in pooled human gamma globulin fractions, but did not demonstrate a rise of antibody titer in acute- and convalescent-phase sera from infantile gastroenteritis patients. The results of serological tests are open to various interpretations. The first possibility is that the same agent was responsible for acute gastroenteritis in infants as well as in older children. The presence of appreciable levels of NT and CF antibodies in acute-phase sera and subsequent pronounced antibody responses in the older children might be indicative of a reinfection with the same agent. This idea is further supported by the fact that immunoglobulin M antibody was found in sera of infants and young children but not in those of older ones (unpublished data). In this regard, our findings seem to coincide with those of Kapikian et al. (7), who found that neither CF antibody nor antibody detected in the indirect fluorescent-antibody procedures was the primary determinant of protection against human rotavirus infec-

tion. It remains to be determined whether a higher level of NT antibody against NCDV, as found in older children, can afford protection. The second possibility is that the causative agents were different, and therefore serological responses to NCDV differed in the two types of acute gastroenteritis. This question cannot be answered until the agents are isolated and identified in cell culture; however, the first interpretation seems to be more plausible. Since sero-epidemiological investigations are in progress on sera from additional cases of acute gastroenteritis and on normal subjects, using the serological tests described here, we will be able to gain more insight into this problem.

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