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Antigenic Relationships among Five Reovirus-Like (RVL) Agents by Complement Fixation (CF) and Development of New Substitute CF Antigens for the Human RVL Agent of Infantile Gastroenteritis (39434)

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In 1974 we described the development of a complement fixation (CF) test for the human reovirus-like (HRVL) agent of infantile gastroenteritis, using as antigen an HRVL particle-rich stool filtrate (1). Later we developed a CF test for the serologically related Nebraska calf diarrhea virus (NCDV) using concentrated cell culture-grown NCDV as antigen (2). We also found that the NCDV could be used as a substitute CF antigen for the HRVL agent as a large proportion of individuals infected with the HRVL agent developed serological evidence of infection not only to the human agent but to the cell culture-propagated calf agent as well (2). Although the NCDV CF antigen was not quite as efficient as the HRVL CF antigen for detecting infection with the HRVL agent, the ready availability of a cell culture-grown source of CF antigen was important for epidemiological studies since the supply of the HRVL antigen was limited because particle-rich stools suitable for CF antigen were found only infrequently. We also observed that the HRVL agent was related by CF to the epizootic diarrhea of infant mice (EDIM) virus (1, 2), the simian agent (SA)-11, and the "O" (offal) agent (2), which was recovered from mixed intestinal washings of sheep and cattle (3).

In this report we describe: (i) the development of a CF test for the SA-11 and the "O" agent; (ii) the antigenic relationships by CF among the five reovirus-like agents in greater detail since antisera to the SA-11 and the "O" agent are now available; (iii) studies which demonstrate that the SA-11, "O", and EDIM viruses can also be used as substitute CF antigens for the HRVL agent; and (iv) studies which reveal that the "O" agent, which grows quite readily in cell cultures, is significantly more efficient than the NCDV as a substitute CF antigen and should therefore be used preferentially when the HRVL agent is not available.

Materials and Methods. CF antigens for the HRVL agent and NCDV, prepared as previously described (1, 2), consisted of 2% stool filtrates containing the HRVL agent (D2, D2A, D3A, Da1A) and bovine embryonic kidney culture-grown 25× concentrated NCDV, respectively. The HRVL suspensions had a titer of 1-64 antigen units in tests with convalescent human sera. Three NCDV antigens had a titer of 1-8 units with convalescent NCDV calf sera; markedly higher titers resulted when 2 of the 3 antigens were also tested with hyperimmune NCDV guinea pig sera. A new lot of EDIM CF antigen which was higher titered (16-64 CF antigen units) than that previously used by us was purchased from Microbiological Associates, Inc.; the new lot was prepared from infected suckling mouse intestines using genetron treatment and centrifugation procedures (4). A CF test for the EDIM virus has been described previously (5). The HRVL agent, NCDV, and EDIM virus antigens were used undiluted in the CF tests to be described. Antisera for these three agents were prepared or obtained as previously described (2). The SA-11 and "O" viruses which had previously been passaged in vervet monkey kidney (MK) cell cultures and an additional six times in primary baboon kidney cell cultures were kindly furnished by Dr. H. H. Malherbe of the Southwest Foundation for Research and Education, San Antonio, Texas. These agents were further passaged in our laboratory in primary vervet (African

green monkey) MK cell cultures maintained with equal amounts of Eagle's medium 2 and medium 199 with glutamine and appropriate antibiotics; serum was not used in the maintenance medium, and cultures were washed once prior to addition of maintenance medium to reduce the amount of residual serum from the growth medium. The "O" and SA-11 viruses induced easily detectable, definite, and reproducible cytopathic effect (CPE) by the fourth day after inoculation. The SA-11 and the "O" agent CF antigens used in this study were prepared as follows: The SA-11 and the "O" agent were passaged in vervet MK cell cultures in our laboratory a total of six and three times, respectively. On the fourth day after inoculation, when CPE was observed over most of the cell sheet, the cultures were frozen. Later the cultures were thawed and shaken on a Vortex mixer, and fluid and cells were harvested and used undiluted as CF antigen. The SA-11 and "O" viruses had infectivity titers of $10^{5.0}$ and $10^{5.5}$ $TCID_{50}$/0.2 ml, respectively. In tests with homologous hyperimmune animal sera the SA-11 and "O" viruses had CF antigen titers of 4–16 and 8–64 units, respectively. Antisera to the SA-11 and the "O" agent were prepared as follows: The SA-11 and the "O" agent were banded on a CsCl density gradient. Based on the knowledge that the serologically related NCDV and HRVL agent had a buoyant density in CsCl of approximately 1.35–1.37 g/cm^3 (2, 6, 7), we combined fractions with a density of 1.371 and 1.352 g/cm^3 from the SA-11 gradient and fractions with a density of 1.357 and 1.335 g/cm^3 from the "O" agent gradient. Each combined fraction was mixed with an equal volume of Freund's complete adjuvant, and 1.0 ml was inoculated intramuscularly into guinea pigs. About 4 weeks later, each combined fraction was mixed with an equal volume of incomplete Freund's adjuvant and 1.0 ml inoculated intramuscularly as a booster. The following control preparations were used in the CF tests to be described in Table I: 2% human stool filtrate, negative for the HRVL agent, prepared from a stool of an infant hospitalized with a respiratory tract illness; uninoculated bovine embryonic kidney cell cultures 25× concentrated; genetron-treated, centrifuged intestines from uninoculated suckling mice; and uninoculated African green MK cell cultures.

Results. Antigenic relationships among the HRVL agent, NCDV, EDIM virus, SA-11, and "O" agent. By CF each of the five reovirus-like agents reacted with its homologous hyperimmune antiserum to a titer which was similar, or in some instances identical, to that observed with each of the heterologous antisera (Table I). In addition, four children who had shed the reovirus-like agent and who had previously developed a serological response to the HRVL agent and NCDV not only again developed a seroresponse to these two viruses but also responded to the SA-11 and "O" viruses; also, three of the four developed seroresponses with the EDIM antigen (Table I). Thus, the five reovirus-like agents could not be differentiated by CF studies with hyperimmune animal or convalescent human sera. However, in tests with paired acute or cord and convalescent sera from two calves which developed illness following challenge with NCDV (8), the NCDV and the "O" agent each reacted with the convalescent serum of one of the calves to a titer of 1:32 whereas the other three reovirus-like agents failed to react with this serum; in addition, the NCDV and the "O" agent each reacted to a titer of 1:16 and the EDIM virus to a titer of 1:8 with the other calf's convalescent serum whereas the HRVL agent and SA-11 again failed to react. Antibody was not detected in the calves' preillness sera by any of the five antigens (<1:20 and <1:8 in such sera from the first and second calves, respectively).

Electron micrographs of the CF antigens used in Table I for the HRVL agent, NCDV, EDIM virus, SA-11, and the "O" agent are shown in Fig. 1. The morphologic similarity of the five reovirus-like CF antigens is striking (6, 10–13).

Serological tests with the five reovirus-like agents and paired acute and convalescent human sera. Since seroresponses were observed with the SA-11, "O," and EDIM viruses in tests with human paired sera, we attempted to compare the efficiency of these agents with that of the HRVL agent and the NCDV for detecting seroresponses in paired sera from infants and young children who had developed diarrhea and also shed the

TABLE I. ANTIGENIC RELATIONSHIPS AMONG THE HRVL AGENT, NCDV, EDIM VIRUS, SA-11, AND "O" AGENT WITH HYPERIMMUNE ANIMAL AND CONVALESCENT HUMAN SERA BY COMPLEMENT FIXATION[a]

Virus	Source of CF antigen[b, c]	Reciprocal of serum antibody titer to indicated virus[d] (source of serum)					Reciprocal of child's acute/convalescent serum			
		HRVL (guinea pig)	NCDV (guinea pig)	EDIM (mice)	SA-11 (guinea pig)	"O" (guinea pig)	Child 1	Child 2	Child 3	Child 4
HRVL	Stool filtrate (Da1A)	640	1024	1280	512	2048	<4/128	<4/64	<4/128	<4/128
NCDV	BEK cell cultures	1280	2048	1280	2048	8192	<4/32	<4/16	<4/32	<4/32
EDIM	Intestines of suckling mice	640	512	320	1024	4096	<4/32	<4/<4	<4/32	4/32
SA-11	African green monkey kidney cell cultures	1280	2048	2560	2048	8192	<4/32	<4/8	<4/16	<4/16
"O"	African green monkey kidney cell cultures	1280	2048	640	2048	4096	<4/32	<4/8	<4/32	<4/16

[a] All results in table obtained in same test.

[b] Titer of each antigen (0.025 ml) as calculated in simultaneous (straight line) titration in this test: HRVL agent, 2 units (tested with two of the convalescent human sera shown); NCDV, 1–2 units (tested with two convalescent NCDV calf sera); EDIM virus, 64 units; SA-11, 16 units; "O" agent, 32 units. EDIM, SA-11, and "O" viruses tested with the homologous hyperimmune animal serum shown. In a subsequent titration which included the same sera plus the hyperimmune animal sera for the HRVL agent and NCDV shown, the titers of the five viruses were as follows: HRVL agent, 2 units with convalescent human sera, 4 units with homologous huperimmune animal serum; NCDV, 8 units with convalescent calf sera and 64 units with hyperimmune animal serum; EDIM virus, 64 units; SA-11, 16 units; and "O" agent, 32 units.

[c] Control antigens described in Materials and methods were employed for each viral antigen and tested against each serum. Antibody was not detected in tests with pre- and postimmunization animal sera; in tests with human paired sera, antibody was not detected with control calf, control murine, and control simian antigens, while with control human stool filtrate antigen, antibody (1:8–1:32) was detected but without significant seroresponses.

[d] Antibody was not detected in any of the preimmunization sera in tests with all five reovirus-like antigens.

HRVL agent. As seen in Table II, of the 35 patients whose paired sera were studied, 26 (74%) developed serological evidence of infection with the HRVL agent, 15 (43%) with NCDV, 18 (51%) with EDIM virus, 20 (57%) with SA-11, and 25 (71%) with the "O" agent. The HRVL agent was significantly more efficient than the NCDV ($\chi^2 = 7.1$, $p < 0.01$) and the EDIM virus ($\chi^2 = 3.9$, $p < 0.05$) but not significantly more efficient than the SA-11 and "O" viruses for detecting a seroresponse. The NCDV was significantly less efficient than the "O" agent ($\chi^2 = 5.8$, $p < 0.02$) but not significantly less efficient than the SA-11 and EDIM viruses. The table also shows that 31 (89.6%) of the 35 patients had a seroresponse to the HRVL and/or "O" agents and that concordance in the detection of the presence or absence of a seroresponse was found in 24 (69%) of the 35 paired sera tested with the HRVL and "O" agents. The geometric mean titers achieved in convalescent sera of seroresponders was significantly higher ($p < 0.001$, t test, two tail) with the HRVL agent than with any of the other four reovirus-like agents (Table II).

Relationship of SA-11, "O," and EDIM viruses to reovirus types 1, 2, and 3 and 20 orbiviruses by CF technique. In further CF tests, the SA-11, "O," and EDIM viruses did not react with a 1:4 dilution of a polyvalent reovirus guinea pig antiserum which reacted to a 1:32 dilution with reovirus types 1 and 2 and to a 1:64 dilution with reovirus type 3. In addition, reovirus types 1, 2, and 3 did not react by the CF technique with 1:8 dilutions of hyperimmune guinea pig sera to SA-11 and "O" viruses or with a 1:10 dilution of hyperimmune sera from mice immunized with EDIM virus. These hyperimmune sera had homologous titers of 1:1024, 1:2048, and 1:320 to the SA-11, "O," and EDIM viruses, respectively. The lack of a relationship of the SA-11 and "O" viruses with the three reovirus types, and the EDIM virus with reovirus type 3, is consistent with previous studies by other techniques (3, 14). The SA-11, "O," and EDIM viruses as well as reovirus types 1, 2, and 3 did not react with polyvalent ascitic fluids from immunized mice which contained CF antibody to 20 orbiviruses previously listed (1, 2).

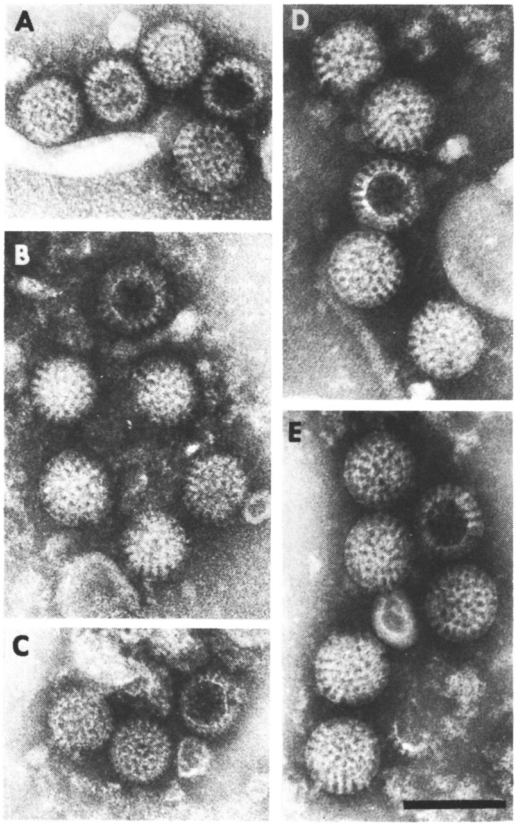

FIG 1. Reovirus-like particles observed in preparations of five viruses used as CF antigens. One milliliter of each reovirus-like CF antigen shown in Table I was centrifuged for 1.5 hr at 17,000 rpm and prepared and examined by electron microscopy by methods similar to those described previously (9). A = HRVL agent; B = NCDV; C = EDIM virus; D = SA-11 virus; E = "O" agent. Preparation of antigens described in Materials and methods. The bar = 100 nm and applies to A through E.

Discussion. We had previously shown that the cell culture-grown and concentrated NCDV could be utilized as a substitute CF antigen for detecting infection with the HRVL agent. The present studies demonstrate that three other related reovirus-like agents, the "O," SA-11, and EDIM viruses may also be used as substitute CF antigens. However, the "O" agent appears to be the most efficient of these four substitute CF antigens and, thus, it should be used preferentially when the HRVL agent is not available. The "O" agent was about as efficient as the HRVL agent and significantly more efficient than the NCDV for detecting seroresponses in patients shedding the HRVL agent. In addition, the "O" agent had another major advantage over the NCDV as a substitute CF antigen: It was easier to prepare since it did not have to be concentrated prior to use as CF antigen; unconcentrated cell culture harvests contained 16–64 units of CF antigen. The availability of the "O" agent as CF antigen should enable the efficient serodiagnosis of disease due to the HRVL agent. The greatest efficiency of detecting serological evidence of infection with the HRVL agent was achieved when sera were tested with both the HRVL and "O" agents as 31 (89%) of the 35 patients developed serological responses to either or both antigens.

TABLE II. NUMBER OF SERORESPONSES DETECTED TO FIVE REOVIRUS-LIKE AGENTS BY CF IN PAIRED SERA FROM 35 DIARRHEA PATIENTS WHO SHED THE HRVL AGENT[a]

Antigen	Reciprocal of geometric mean titer in convalescent serum of seroresponders[b]	Patients who developed a seroresponse to indicated antigen (No. (%))	Patients who developed a seroresponse when results of CF tests with indicated pairs of antigens were combined (No.)				
			HRVL	NCDV	EDIM	SA-11	"O"
HRVL	28.0	26 (74%)	–	27	28	27	31
NCDV	15.3	15 (43%)	27	–	23	20	25
EDIM	10.5	18 (51%)	28	23	–	24	26
SA-11	12.1	20 (57%)	27	20	24	–	25
"O"	11.8	25 (71%)	31	25	26	25	–

[a] Fourfold or greater rise in CF antibody titer in paired sera.

[b] In acute phase serum of seroresponders, antibody was not detected (<1:4) in 23 of 26 with seroresponses to HRVL agent, in 14 of 15 with seroresponses to NCDV, in 17 of 18 with seroresponses to EDIM virus, in any of 20 with seroresponses to SA-11 virus, and in 24 of 25 with seroresponses to the "O" agent. With one execption, the maximum titer used in calculating the geometric titer (GMT) was 1:32 even though some convalescent sera had higher titers; 1:32 was used since all convalescent sera of seroresponders were titered at least up to this dilution. In the exception (with NCDV antigen) a single convalescent serum with a 1:64 titer was included since the acute serum of this pair had a 1:16 titer and to qualify for a seroresponse a fourfold or greater rise in antibody had to be observed (tabulating this serum as 1:32 resulted in a GMT of 1:14.6).

The finding of additional substitute CF antigens for the HRVL agent may also have important implications in the immunoprophylaxis against human infantile gastroenteritis caused by the HRVL agent (15, 16). Thus, efforts to find suitable strains of reovirus-like agents in various species should be pursued as it may be possible to immunize against the human disease utilizing an animal reovirus-like strain if one of these strains proves able to infect man without causing illness. Of course, attempts to find naturally occurring attenuated strains of the human agent or to develop such attenuated strains should be pursued as well as attempts to develop inactivated vaccines once the HRVL agent is grown efficiently in culture (17–20). Further studies will determine the course which should be pursued for immunoprophylaxis of this disease.

Summary. The human reovirus-like (HRVL) agent, Nebraska calf diarrhea virus (NCDV), epizootic diarrhea of infant mice (EDIM) virus, simian agent (SA)-11, and the "O" (offal) agent were found to be similar, if not identical, in reciprocal complement fixation (CF) tests employing hyperimmune animal sera. In addition, in CF tests with paired sera from 35 diarrhea patients who shed the HRVL agent, 74% developed serologic evidence of infection with the HRVL antigen, 43% with NCDV, 51% with EDIM virus, 57% with SA-11, and 71% with the "O" agent. Thus, in addition to the NCDV, which had previously been described as a suitable substitute CF antigen for the HRVL agent, the SA-11, "O," and EDIM viruses may also be utilized as substitute antigens for the HRVL agent. However, the "O" agent appears to be the most efficient of the four substitute CF antigens and thus should be used preferentially when the HRVL agent is not available. The "O" agent was about as efficient as the HRVL agent and significantly more efficient than the NCDV for detecting seroresponses. The greatest efficiency for detecting infection with the HRVL agent resulted when sera were tested with both the HRVL and "O" agents as 31 (89%) of the patients developed serologic evidence of infection with one or both antigens. The finding of additional substitute CF antigens for the HRVL agent may have implications in the immunoprophylaxis against human disease.

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