PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 145, 366–370 (1974)

RD-114 Virus Infectivity Assay by Measurements of DNA Polymerase Activity and Virus Group Specific Antigen[1] (37812)

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(Introduced by E. H. Lennette)

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Infectivity of RD-114 virus (RDV), an endogenous cat type-C virus (1–3), for human cells was first demonstrated when cell cultures exposed to the virus were shown by electron microscopy to contain type-C virus particles, gave positive interspecies group specific antigen reaction in immunodiffusion tests, and released RNA-dependent DNA polymerase activity into the culture fluids (4). Thereafter, Oroszlan *et al.* (5) purified the major internal protein, gs-1, of RDV and used it to prepare antibody in guinea pigs. As for infectivity assays of nontransforming type-C RNA viruses of chickens, mice, hamsters, and cats (6–8), such antibody could be used to detect induction of RDV specific gs-1 antigen in infected cells. In addition, Kelloff, Hatanaka and Gilden (9) reported an assay of type-C virus infectivity by measurement of RNA-dependent DNA polymerase activity. This paper describes the assay of RDV infectivity by measurements of gs-1 antigen and of polymerase activity induced in inoculated cultures. It compares these assays with those based upon the capacity of RDV to cause a syncytial cytopathic effect upon human KB or KC cells (10, 11).

Materials and Methods. The RDV used in these experiments was the tissue culture fluid from the 23rd to the 55th *in vitro* tissue culture passage of the cell line XC-114B (12) harvested 24 hr after the fluid change and clarified by filtration through a Millipore (0.45 μm) filter. Cell-free extracts of RDV were also prepared from pelleted XC-114B cells by the Moloney procedure (13).

The human rhabdomyosarcoma cell line, RD (14), used for the infectivity assays (in 33rd to 76th passage level), was propagated in Eagles minimum essential medium (EMEM) supplemented with 10% heated bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and glutamine (2 m*M*), and was passed by trypsin dispersion once a week.

RD cells, trypsinized and resuspended in growth medium, were plated in 25 or 75 cm^2 Falcon plastic flasks, 3.5×10^5 or 1.5×10^6 cells/flask, respectively. Twenty-four hours after plating, the fluid was removed and 0.4 ml (per 25 cm^2 flask) or 1.2 ml (per 75 cm^2 flask) of virus inoculum was added. After 1 hr incubation at 37°, 4 or 15 ml of complete growth medium was added. Thereafter, the fluid was changed every 2 or 3 days. The infected cultures were subcultured to two new flasks approximately every 10 days.

Fluids were harvested for polymerase assay at 24 hr, clarified (17,000*g*, 10 min), pelleted (81,000*g*, 90 min), and resuspended in 0.04% NP-40, 20 m*M* dithiothreitol. Reaction mixtures (50 μl total) contained 25 μl of resuspended sample, 2

[1] This study was conducted under contract PH 43-68-1030 within the Special Virus-Cancer Program of the National Cancer Institute, NIH, PHS.

[2] We thank Mrs. Mary Peer, Mrs. Mary Ann Gaffey, and Mrs. Marie Krempin for technical assistance.

TABLE I. Assay of RDV (Pool No. 2) by DNA Polymerase Activity and by Group Specific Antigen Induction.

	Days:	1		3		5		8		17		24	
RDV		RDP[a]	gs-Ag[b]	RDP	gs-Ag	RDP	gs-Ag	RDP	gs-Ag	RDP	gs-Ag	RDP	gs-Ag
10^{0}		0[c]	<1[d]	4579	<1	8372	<1	18,537	<1	—	—	—	—
10^{-1}		5	<1	245	<1	1831	<1	18,834	1	57,921	4	136,121	2
10^{-2}		6	<1	64	<1	77	<1	2051	<1	44,054	4	29,670	2
10^{-3}		—[e]	—	0	<1	0	<1	258	<1	42,622	4	41,564	4
10^{-4}		—	—	—	—	0	<1	0	<1	14,899	2	113,714	4
10^{-5}		—	—	—	—	0	<1	0	<1	3548	<1	59,908	2
10^{-6}		—	—	—	—	—	—	2	<1	0	<1	0	<1
10^{-7}		—	—	—	—	—	—	—	—	0	<1	0	<1
10^{-1} heated 56°													
30 min		—	—	—	—	—	—	1	<1	168	<1	258	<1
RD cells		0	<1	—	—	—	—	0	<1	103	<1	0	<1

[a] RNA dependent DNA polymerase activity. See Methods.

[b] RDV gs antigen titer using guinea pig antibody to electrofocus purified gs protein.

[c] Incorporation of ^{3}H-TMP. Oligo dT:poly rA as template. TCA precipitable dpm per ml original culture medium. Control dpm (no template) subtracted from each.

[d] Reciprocal of highest dilution giving 3 to 4+ complement fixation.

[e] — = not done.

m*M* $MnCl_2$, 50 m*M* KCl, 40 m*M* Tris (pH 8.1), 2.5 μCi TTP-Me-^{3}H (18 Ci/mmole), and either 0.025 OD_{260} oligo dT·poly rA or water (blank). After 60 min at 37°, aliquots were adsorbed to 24 mm Whatman No. 1 filters prewetted with 10 μl ATP (10 m*M*) and precipitated in cold 5% TCA, 2% sodium pyrophosphate (10 ml/filter). The filters were washed successively in cold 5% TCA–1% sodium pyrophosphate, 5% TCA (2×), 95% ethanol (2×), and ether, dried and counted in a Nuclear Chicago Mark II scintillation counter.

Cell pack antigens to be tested for induction of RDV gs antigen were prepared by scraping the cells from the surface of the flasks with a rubber policeman, centrifuging them at 1000 rpm for 10 min, resuspending to give a 50% (v/v) cell pack, and sonicating for 2 sec at 4 setting on a Branson sonicator.

Results. The time course of development of DNA polymerase activity and gs antigen in infected cultures is summarized in Table I, which presents a representative example of 11 such assays, carried out 7 to 35 days after infection of RD cells. No polymerase activity or gs antigen were detected 24 hr after infection; 3 and 5 days after infection, polymerase activity, but no gs antigen, was detected; 8, 17, and 24 days after infection, both polymerase activity and gs antigen were detected in increasing levels in the infected cultures. The highest infectivity titer (10^7 infectious doses/0.4 ml) of RDV (pool No. 2) was obtained 28 and 35 days after infection of RD cells (data not shown). Electron microscopy of pellets of the infected cells revealed no type-C virus particles 1 day after infection but they were present 3 days after infection.

TABLE II. Virus Neutralization of RDV.

RDV incubated with[a]		RDP[b]	RDV[c] gs-Ag
EMEM with 10% fetal bovine serum		2105[d]	4[e]
Normal rabbit serum	1:10	868	4
Rabbit RDV antiserum	1:10	0	<1
	1:20	0	<1
	1:40	69	<1
	1:80	917	2
	1:160	570	2

[a] Tissue culture fluid pool diluted 1:10 from RDV culture was incubated with equal amounts of respective dilutions of antiserum, control serum, or medium for 1 hr at room temperature before inoculation.

[b–e] See Table I. Assays carried out 7 days after infection of RD cells.

TABLE III. Inactivation of RDV-Induced DNA Polymerase Activity and gs Antigen by Heat and Chloroform.

Inoculum	RDP[a]	RDV gs-Ag[b]
None—RD cells	0[c]	<1[d]
RDV 10^{-1}	10,772	4
RDV 10^{-1} heated 56° 30 min	13	<1
RDV 10^{-1} chloroform treated	142	<1

[a–d] See Table I. Assays carried out 7 days after infection of RD cells.

With these techniques it was demonstrated that the capacity of RDV to induce polymerase activity and gs antigen was neutralized by specific rabbit antiserum (Table II) (R. M. McAllister and M. O. Nicolson, unpublished data) and was inactivated by heating at 56° for 30 min and by treatment with chloroform (Table III). No polymerase activity or gs antigen appeared in RD cells inoculated with supernatant fluids from RD cells, KB or HeLa cells, or cells derived from another rhabdomyosarcoma (Cope cells), or three osteosarcomas (MT, Mar, Kief cells). It was possible to pass the RDV released (polymerase activity and gs antigen inducing capacity) from the infected RD cell cultures by cell-free extracts of cell suspensions and by filtered culture fluids.

The heat and chloroform sensitivity, as well as the neutralization by specific antiserum prepared by immunization of the rabbits with RDV purified on sucrose density gradients (fractions with specific densities 1.16 g/ml), indicate that the polymerase and gs antigen inducing agent has the same physicochemical and immunological characteristics as RDV (4, 10).

Table IV compares the RDP and gs antigen induction assay methods with two other published methods (10, 11). Approximately the same infectivity titer is obtained by all four methods when the assays are carried

TABLE IV. Comparison of RDV Assay Systems.

Assay system	RDV culture fluids		RDV cell-free extract
	Pool 1	Pool 2	
RDV-gs-Ag induction[a]	10^{-1}[c]	10^{-2}	10^{-3}
RDP[b]	10^{-1}	10^{-1}	10^{-3}
Syncytium induction in KB cells[d]	10^{-1}	ND	10^{-2}
Syncytium induction in KC cells[e]	10^{-1}	10^{-1}	10^{-2}
Electron microscopy (type-C particles/ml)	ND	10^{9}	$<10^{8}$

[a,b] See Table I.

[c] Highest virus dilution causing induction of RDV gs antigen and RDP 7 days after infection of RD cells. See text for details.

[d] See Ref. (10); syncytia counted 8 days after infection of KB cells.

[e] See Ref. (11); syncytia counted 9 days after infection of RD cells, 2 days after overlay with KC cells.

out 7–9 days after infection of RD or KB cells according to the latter two methods. The KB and KC cell assay systems are simpler than the other two systems; however, the combined use of the RDP and gs antigen induction assays carried out 21–28 days after infection of RD cells give a more accurate estimate of the infectivity titer (10^{7} infectious doses/0.4 ml for pool No. 2) than do the other assay systems. Of interest, the physical virus particle to infectious dose ratio for RDV in pool 2 was about 10^{2}.

Discussion. Although evidence of RDV infection of RD cells is present 3 days after infection by detection of DNA polymerase activity and observable type-C virus in the infected cells, approximately 3–4 wks and one or two subcultures of the infected cells are required to determine the infectivity of higher dilutions of virus. A similarly slow progressive infection of human lymphoblastoid cell cultures has been observed (15); the explanation for these observations is unknown.

In contrast to our findings, Riggs *et al.* (unpublished data) could detect newly formed RDV gs-1 antigen by an indirect immunofluorescent technique in a line of dog cells 24 hr after infection. This result suggests that this technique was more sensitive for the detection of gs-1 antigen than the CF test used by us; however, studies of RDV-infected RD cells using it were not carried out.

During the course of these studies, many human cell strains and lines other than RD cells, as well as nonhuman cell strains, were surveyed for their susceptibility of RDV using the RDP and gs antigen induction assays 21 days after infection. None, including epithelial cell lines (HeLA, KB, J-111), sarcoma cell strains (Cope, MT), human, or beagle embryo fibroblasts, was as susceptible as RD cells. These data will be reported separately. These observations, as well as the observed capacity of RD cells to rescue the Crandall virus (feline endogenous type-C virus) (1–3) and the AT-124 virus (NIH Swiss mouse endogenous type-C virus) (16), suggest that RD cells are notably permissive for two endogenous type-C viruses. The explanation for this permissiveness may be of interest and is being sought.

Summary. The infectivity of RDV, a nontransforming type-C virus, can be assayed by induction of virus-specific gs antigen and of DNA polymerase activity in infected cell cultures. RD cells are more susceptible to RDV infection than other human cell lines or strains tested. Although RDV infection of the cells can be detected 3 days after exposure of RD cells to large doses of virus, 3–4 wk are required to obtain demonstrable infection of cell cultures infected with small doses of virus. Simpler assays based upon the capacity of RDV to induce syncytia in KB or KC cells 7 to 9 days after infection expectedly yield lower infectivity titers than those obtained by RDP or gs antigen induction assays in RD cells 21–28 days after infection.

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Received Sept. 4, 1973. P.S.E.B.M., 1974, Vol. 145.