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Studies on the Prevalence of Endogenous Type C Virus RD-114 in Cats[1] (37890)

PADMAN S. SARMA, AMELIA SHARAR, JOAN TSENG, PAUL J. PRICE, AND MURRAY GARDNER

National Cancer Institute and Microbiological Associates, Bethesda, Maryland and University of Southern California, Los Angeles, California

The RD 114 virus was initially discovered in a cell line of human rhabdomyosarcoma (RD) (1) after passage through a cat fetus (2). This virus, continuously released by the RD cells, was found to be distinct from any of the previously known type C viruses of various species, in antigenic and biologic characteristics (3–6), thus suggesting the possibility that the virus may have been activated from RD cells during its passage through cat (2–6). Our recent virological studies (7) and similar studies of other investigators (8, 9) showed that a virus similar to RD 114 can be evoked from apparently normal cells of an established kidney cell line derived from a normal cat (10). Other studies provided biochemical evidence suggesting the presence of RD 114 viral DNA and RNA in normal tissues as well as tumor tissues derived from cats (11–15).

In this communication, we provide additional evidence which confirms the widespread prevalence of RD 114 viral genome in normal cats. Feline cells of diverse origin were found to contain the RD 114 virus in a "hidden" and activatable form; the virus was detectable in an infectious form in certain fetal cat tissues and in the tissues of cat XC 114B through which the RD cell line has been passaged as described above (2). Our studies conclusively prove the cat derivation of the RD 114 virus and the original mode of infection of the RD cell line with this virus (2).

Materials and Methods. Tissue cultures. Feline embryo fibroblast (FEF) cell cultures used for the propagation and isolation of feline leukemia viruses (FeLV), were used between the 2nd and 10th *in vitro* passage levels as described (7, 16). These cultures and RD cell cultures were propagated in disposable Falcon petri dishes in a medium consisting of Eagle's minimum essential medium supplemented with 2 mM L-glutamine, 10% fetal calf serum (heated at 56° for 30 min) and antibiotics at the concentration of penicillin 250 units/ml, streptomycin 250 μg/ml, Fungizone 2.5 μg/ml, and Kantrex 50 μg/ml. The cultures were incubated in a 37° CO_2 incubator flushed with 5% CO_2 in air. The cultures were transferred by trypsinization (17) at weekly intervals.

Cat cell cultures. "Virus-free" cat cell cultures, free of demonstrable group-specific (gs-1) antigens of RD 114 virus (2, 3) and FeLV (16, 18) were tested for the presence of "inducible" covert type C viral genome by the techniques described below. The cultures were derived from diverse embryonic and adult cat tissues from three sources. The cultures examined included a subline CRFK of Crandell cat cells and several single cell clones derived from our subline C-C (7) of the same Crandell cat cells (10). These clones were prepared by the method described by Freeman *et al.* (19).

RD cultures. Virus-free cultures of RD cells, which we found to be the most sensitive for *in vitro* propagation and assay of RD 114 virus (7, 20) were used between *in*

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vitro passage levels of 67 and 77 for the detection and assay of RD 114-like viruses recovered in this experiment.

Virus detection. Specimens suspected to contain virus were inoculated into RD cell cultures for the isolation of the RD 114 virus and into FEF cultures for the isolation of FeLV. The inoculated cultures and parallel control cultures were maintained for 3 weeks. Cell antigens were then collected and tested in the complement-fixation (CF) test for the presence of the group-specific (gs-1) antigens of RD 114 (2, 3) and FeLV (16, 18). Virus isolation was confirmed by serial passages of culture fluids in homologous cultures.

Virus-induction experiments. Freshly plated feline cultures were exposed to 30 μg/ml of 5-iododeoxyuridine (IdU) for 48 hr as previously described (7). The cultures were then trypsinized and co-cultivated with equal parts of RD cells or without RD cells (7). Culture fluids of mixed cultures were collected on the 7th day after mixed cultivation, clarified, and tested for infectious virus as described above. After 21 days of mixed cultivation, cell antigens of mixed cultures were tested in the CF test for the presence of gs-1 antigens of RD 114 (2, 3) and FeLV (16, 18).

Direct virus isolation from cat tissues. The following specimens, stored in liquid nitrogen for varying periods of time, were tested for infectious RD 114 virus and/or viruses of the feline leukemia/sarcoma group: (a) 10% crude extracts of thymus and liver of cat fetus CF 72 and a similar extract of thymus derived from cat fetus CF 81-B derived from another cat, all of which contained RD 114 gs-1 antigen in titers of 1:2 to 1:8; (b) crude 10% extract of spleen and bone marrow derived from kitten XC 114B [thin sections of tissue made at the time of necropsy showed the presence of type C virus particles (1)]; (c) clarified 10% extract of a spleen of a normal adult cat; (d) 14 clarified 10% extracts of lymphosarcoma tissues derived from 12 young cats with naturally occurring neoplasia.

Identification of selected virus isolates. The RD 114-like virus recovered from the spleen and bone marrow samples of cat XC 114B was purified from the accompanying FeLV by serial passages of the virus in RD cell cultures at the highest virus dilution capable of inducing the RD 114 gs-1 antigen in this culture. A virus neutralization test was then performed to determine envelope antigenic relatedness of these virus isolates to the RD 114 virus. A potent RD 114

TABLE I. Virus-free Feline Cells of Diverse Origin Found to Release RD 114-like Virus After Treatment with 5-Iododeoxyuridine and Cocultivation with Human Rhabdomyosarcoma Cells, RD.

Origin	Tissue culture of	Cell designation	Passage no.
Embryo	Whole embryo	3402	1
	Whole embryo	3416	1
	Whole embryo	FFc WF	5
	Whole embryo	D843	12
	Fetal tongue	FFc 3Tg	7
	Fetal thymus	FFc 2Th	7
Adult	Osteosarcoma	F 47	137
	Osteosarcoma	F 100	76
	Adult kidney	Crandell subline CRFK	176
	Adult kidney	Crandell subline C-C (7 clones)	5 to 9[a]

[a] Cell clones were established at 220 *in vitro* passage level of subline C-C. Clones were tested within four to nine passage levels after establishment as clones.

virus-neutralizing antiserum prepared in guinea pigs ($\log_{10}$ neutralization index > 3) was mixed with equal parts of virus, diluted so as to contain 100 CF antigen infectious units per 0.2 ml of virus–serum mixtures. The virus–serum mixtures were incubated at 4° for 40 min and then tested for residual virus by inoculation in 0.2-ml amounts in RD cultures.

Results. Prior to application of IdU, the 16 cat cell cultures derived from nine cats were free of infectious RD 114 virus, FeLV and their respective gs-1 antigens. However, on application of IdU to cat cultures and subsequent co-cultivation with RD cells, RD 114-like viruses were recovered in infectious form from every one of 16 cat cell cultures we examined (Table I). These included six cultures of diverse normal embryonic tissues from six cats, cultures of tumor (osteosarcoma) of two adult cats, one subline CRFK of Crandell cat cell line, and seven single cell clones we prepared from subline C-C of the same Crandell cat cell line. A CF test done on mixed-culture antigens collected on the 20th day showed the presence of RD 114 gs-1 antigen (titer $>$ 1:4). FeLV gs-1 antigen was also detected in six cat cultures derived from different cats. The FeLV CF titers ranged from 1:2 to 1:4. However, this antigen was not detectable by CF test done on cell antigens examined on the 40th day. In addition, our attempts to isolate an infectious FeLV by passage of culture fluids into FEF and RD cultures gave inconclusive or negative results.

Results of direct tests of cat tissue extracts for virus are summarized in Table II. One of two cat fetus thymus specimens which contained RD 114 gs-1 antigen yielded one isolate of an RD 114-like virus in RD cultures. The spleen and bone marrow specimens of cat XC 114B, previously found to contain type C virus particles (1), contained infectious FeLV as well as infectious RD 114-like virus capable of replicating in the inoculated RD and FEF cultures. The viruses were separated from each other by the described terminal dilution technique. The RD 114 virus isolates derived from cat XC 114B were neutralizable by guinea pig antiserum we prepared against the RD 114 virus derived from RD 114 virus-producing RD cell line (2).

FeLV was isolated from the spleen extract of a normal adult cat. Fourteen cat lymphosarcoma extracts also yielded eight isolates of FeLV. RD 114 virus was not recovered from any of these lymphoma specimens.

Discussion. Our studies reveal that cat cell cultures from diverse embryonic and adult tissues and sources contain covert RD 114 virus in an inducible form. Thus, eight cultures derived from eight cats, a subline CRFK of Crandell cat cells, and seven single cell clones we prepared from subline C-C (7) of the same Crandell cat cells yielded RD 114-like virus isolates upon induction with IdU. The induced virus replicated and established productive infection of RD cultures in a manner we previously found for a similar virus we induced from virus-free sublines C-C and C-H of Crandell cat kidney cell line (7). The induction of RD 114-like virus from virus-free single cell clones derived from an "inducible" C-C subline of Crandell culture (7) suggests that all cells of the Crandell cat cell line carry the RD 114 viral genome.

The transient induction of FeLV gs-1 antigen but not infectious virus in induced mixed cultures of cat cells and RD cells suggests that the FeLV genome may be vertically transmitted in a covert form and is consistent with our finding of FeLV gs-1 antigen in cat fetal tissues demonstrable by CF tests and radioimmunoassay (Sarma, P. S., Gardner, M., Parks, W., and Huebner, R. J., unpublished observations). Studies are in progress to confirm and extend these observations.

Our studies, reported herein, conclusively establish the presence of RD 114 virus in the tissues of cat XC 114B through which the RD cells were passaged (2) and thus provides unequivocal evidence on the original mode of infection of RD cells with this virus. The RD 114 virus recovered replicated *in vitro* in RD cells as well as FEF cells. FEF cultures can be infected only with large infectious doses of RD 114 virus (20). The replication in FEF of RD 114 and accompanying FeLV, we observed, may be

TABLE II. Isolation of Feline Leukemia and/or RD 114-like Viruses from Cat Fetal and Adult Tissues.

Origin[a]	Specimen	Virus isolated[b]	
		FeLV	RD 114-like
Embryo	Cat CF 72		
	Fetal thymus	0	1
	Fetal liver	0	0
	Cat CF 81-B		
	Fetal thymus	0	0
Adult	Cat XC 114B		
	Spleen	1	1
	Bone marrow	1	1
	Cat CT 363		
	Normal spleen	1	0
	Cat CT-425		
	Spleen	1	0
	Lymph node	1	0
	Cat CT-172		
	Lymphoma	1	0
	Cat CT-104		
	Lymphoma	1	0
	Cat CT-142		
	Lymphoma	1	0
	Cat CT-498		
	Lymph node	1	0
	Cat CT-538		
	Liver	1	0
	Cat CT-130		
	Lung	1	0
	Cat CT-552		
	Lymph node	0	0
	Cat CT-419		
	Liver	0	0
	Cat CT-460		
	Pancreas	0	0
	Liver	0	0
	Cat CT-348		
	Liver	0	0
	Cat CT-125		
	Lymphoma	0	0
Total isolations		11/20[c]	3/20

[a] With the exception of cats CT-363 and XC 114B, tissues of other adult cats had gross and/or histopathological evidence of lymphoma.

[b] FeLV = feline leukemia virus. See text for virus isolation methods.

[c] Numerator denotes number of specimens positive for virus; the denominator, the total number of specimens from 16 cats tested.

due to the presence of large amounts of RD 114 virus in the specimens we tested or due to phenotypic mixing of RD 114 genome with FeLV envelope, a phenomenon known to occur in mixed infections with enveloped viruses (21). FeLV present in these specimens did not infect the RD cells. We are now attempting to characterize the envelope antigenic type and host range of this FeLV.

The presence in infectious form of RD

114 virus, recoverable without chemical activation, in postnatal cat tissues is unique and has been observed only in the present studies of cat XC 114B. It is conceivable the introduction of RD cells into cat XC 114B during its fetal life, initially activated the endogenous RD 114 virus. Upon infection of RD cells with this virus, the RD cells may have served as a reservoir for the continuous presence of the virus in the tissues of cat XC 114B.

The isolation of RD 114-like virus from a cat thymus CF-72 suggests that the virus can occur in infectious form in cat fetuses under certain circumstances. This observation has been confirmed by Rasheed *et al.* (22). However, the presence of infectious RD 114 virus in cats does not seem to be widespread since screening of cat fetus and adult tissue for RD 114 gs-1 antigen only rarely reveals the presence of this antigen in such specimens (Huebner, R. J., Sarma, P. S., and Gardner, M., unpublished observations). Such rare gs-1-positive specimens include extracts of tumors of old cats which are presently under study to determine the possible presence of "infectious" RD 114 virus.

The general absence of RD 114 gs-1 antigen in adult cat tissues suggests that, under natural conditions, viral proteins are not usually synthesized despite the fact that viral RNA and DNA are widespread in normal cat cells (11–15). This is also supported by our observation on the absence of demonstrable RD 114 virus-neutralizing antibodies in adult domestic cats with or without neoplasia (7).

Since we recovered RD 114 virus from every cat culture we studied, the induction and isolation of RD 114-like virus from cultured "virus-free" feline osteosarcoma cells does not necessarily imply an etiological role of this virus in this naturally occurring neoplastic disease of cats. The role of RD 114 virus in the naturally occurring cancers of cats remains to be determined.

Summary. Virus-free cultures of diverse cat cells derived from normal or tumor tissues contained covert RD 114-like viruses inducible with 5-iododeoxyuridine (IdU). Infectious virus was isolated from each of 16 cat cell cultures derived from nine cats, which included six cultures of diverse, normal embryonic tissues from six cats, cultures of tumor (osteosarcoma) of two adult cats, one subline CRFK of Crandell cat cell line, and seven single cell clones we prepared from subline C-C of the same Crandell cat cell line.

RD 114 virus as well as feline leukemia virus (FeLV) were recovered from the spleen and bone marrow of cat XC 114B through which McAllister *et al.* (1) passed RD cells immediately prior to the original discovery of the RD 114 virus. RD 114-like virus was also isolated from a thymus of a cat fetus. Many cat lymphosarcoma specimens contained FeLV but none contained infectious RD 114.

These studies provide conclusive evidence of the original mode of infection of RD cells with RD 114 and suggest the widespread prevalence of an inducible RD 114 virus genome in the cat population.

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1. McAllister, R. M., Nelson-Rees, W. A., Johnson, E. Y., Rongey, R. W., and Gardner, M. B., J. Nat. Cancer Inst. **47**, 603 (1971).

2. McAllister, R. M., Nicolson, J., Gardner, M. B., Rongey, R. W., Rasheed, S., Sarma, P. S., Huebner, R. J., Hatanaka, M., Oroszlan, S., Gilden, R. V., Kabigting, A., and Vernon, L., Nature (London) **235**, 3 (1972).

3. Oroszlan, S., Bova, D., White, M. H., Toni, R., Foreman, C., and Gilden, R. V., Proc. Nat. Acad. Sci. USA **69**, 1211 (1972).

4. Gilden, R. V., Lee, Y. K., and Long, C. W., Int. J. Cancer **10**, 458 (1972).

5. Long, C., Sachs, R., Norvell, J., Huebner, R. J., Hatanaka, M., and Gilden, R. V., Nature (London) **241**, 147 (1973).

6. Scolnick, E., Parks, W., Todaro, G. J., and Aaronson, S., Nature (London) **235**, 35 (1972).

7. Sarma, P. S., Tseng, J., Lee, Y. K., and Gilden, R. V., Nature (London) **244**, 56 (1973).

8. Livingston, D. M., and Todaro, G. J., Virology **53**, 142 (1973).

9. Fischinger, P. J., Peebles, P. T., Nomura, S., and Haapala, D. K., J. Virol. **11**, 978 (1973).

10. Crandell, R., Fabricant, C. G., and Nelson-Rees, W. A., In Vitro, in press.

11. Ruprecht, R. M., Goodman, N. C., and Speigelman, S., Proc. Nat. Acad. Sci. USA **70**, 2176 (1973).

12. Baluda, M. A., and Roy-Burman, P., Nature (London) New Biol. **244**, 59 (1973).

13. Okabe, H., Gilden, R. V., and Hatanaka, M., Nature (London) New Biol. **244**, 54 (1973).

14. Gillespie, D., Gillespie, S., Gallo, R. C., East, J. L., and Dmochowski, L., Nature (London) New Biol. **244**, 51 (1973).

15. Neiman, P. E., Nature (London) New Biol. **244**, 62 (1973).

16. Sarma, P. S., Gilden, R. V., and Huebner, R. J., Virology **44**, 137 (1971).

17. Sarma, P. S., Turner, H. C., and Huebner, R. J., Virology **23**, 313 (1964).

18. Oroszlan, S., Huebner, R. J., and Gilden, R. V., Proc. Nat. Acad. Sci. USA **68**, 901 (1971).

19. Freeman, A. E., Ward, T. G., and Wolford, R. G., Proc. Soc. Exp. Biol. Med. **116**, 339 (1964).

20. Sarma, P. S., *in* "Perspectives in Virology" (M. Pollard, ed.), Vol. 8, p. 112, Academic Press, New York (1973).

21. Vogt, P. K., Virology **32**, 708 (1967).

22. Rasheed, S., and Gardner, M., in preparation.

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