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RNA-Dependent DNA Polymerase Activity in Canine Lymphosarcoma*

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Abstract—*An assay for RNA-dependent DNA polymerase (RDDP) was used as a screening test for retrovirus expression in canine lymphosarcoma tissue. Enzyme activity of borderline significance was detected with a poly (rC): oligo (dG) template in the supernatants of 3 out of 14 short term cultures of lymphosarcoma cells. Induction with halogenated pyrimidines was not obligatory to detect this activity.*

In crude preparations of tumour tissue RDDP activity was detected in 2 of 11 cases. The inability to detect enzyme activity with a poly (dA): oligo (dT) template in the culture supernatants or in the tumour preparations, excluded the possibility that there was significant contamination with terminal transferase or cellular DNA polymerases. Confirmatory evidence that particles with retroviral properties were present in the two positive tumour preparations was provided by positive culture supernatant RDDP activity or by the presence of RNA containing particles of oncoviral density. However no oncoviruses were isolated by cocultivation with canine kidney cells (MDCK). In the household of one of the RDDP positive cases a second case of lymphosarcoma had recently occurred in an unrelated dog.

INTRODUCTION

In dogs lymphosarcoma is a common cancer of unknown aetiology [1–3]. In several domestic animal species including cats [4] and cattle [5, 6] an exogenous oncoviral aetiology has been established for some lymphoid neoplasia; whilst in pigs endogenous oncoviruses have been found in association with the tumours [7]. Attempts to demonstrate a viral aetiology for the disease in dogs have relied on electron microscopy of tumour tissue and cell free transmission experiments. The limited electron microscopy surveys of canine lymphosarcoma tissue have produced conflicting results. Some surveys have found oncovirus-like particles present at low density in some tumours [8, 9] whilst another survey failed to find comparable particles [10]. Successful cell free transmission of canine lymphosarcoma has not been reported [11] although the rare condition of canine mast cell leukaemia has been transmitted on two separate occasions [12, 13].

An herpes virus has been isolated from one case of canine lymphosarcoma. The isolate produced lymphoid hyperplasia in neonatal dogs but progression to neoplastic disease did not occur [14].

In the experiments described here an assay for RDDP activity has been used as a preliminary screening test for overt retroviruses in canine lymphosarcoma tissue.

MATERIALS AND METHODS

Short-term cell cultures

Surgically removed lymph nodes from histologically confirmed cases of lymphosarcoma were used to prepare short-term cultures. Within a few minutes of excision the nodes were diced in RPMI 1640 medium with 20% FCS and a single cell suspension prepared by gently pressing the tissue through a grade 50 mesh. Damaged cells were removed by centrifugation for 15 min at 1500 *g* on a 5% Ficoll, 8.5% Hypaque gradient. Cells were removed from the Ficoll medium interface, washed once with medium and finally resuspended in 20 ml cultures at 10^6 cells/ml in RPMI plus 20% FCS in a 95% air, 5% CO_2 atmosphere

Some cultures were treated with either IDU or BrDU at 20 μg/ml for 24 hr. At the

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Abbreviations: FCS—Foetal calf serum; IDU—Iododeoxyuridine; BrDU—Bromodeoxyuridine; RDDP—RNA-Dependent DNA polymerase.

end of this period the cells were resuspended in fresh medium without halogenated pyrimidines. Cultures treated with these agents were maintained for a further 4 days before analysing the supernatants for RDDP activity. Untreated cultures were assayed for RDDP activity 5 days after being established.

Cocultures of lymphosarcoma monolayer cells

Cocultures of canine lymphosarcoma cells and monolayer cells were established by plating 2.5×10^6 lymphosarcoma cells in 10 ml RPMI plus 20% FCS on subconfluent monolayer cells in 25 cm^2 culture flasks. Tumour cells were cultured with the canine kidney cell line MDCK which showed no spontaneous or IDU inducible oncovirus release. The cocultures were fed twice a week and the monolayer cells split when confluent. Any surviving lymphoid cells were added back to the cultures. The supernatants from these cocultures were assayed for RDDP activity at weekly intervals for 3 weeks.

RDDP assay

Medium from cell cultures was prepared for RDDP assay by clarification at 3000 ***g*** for 10 min and then at 10,000 ***g*** for 10 min. The clarified medium was layered over 20% glycerol (pH 7.8) and centrifuged for 1 hr at 98,000 ***g***. The resulting pellet was resuspended to 1% of the original medium volume in 40 mM. Tris–HCl pH 7.8.

Tumour tissue was prepared as follows. Approximately 20 g of tumour tissue was homogenised in three volumes of ice-cold TNE buffer (0.01M Tris–HCl pH 8.3, 0.15 N NaCl, 0.01M EDTA) for 1 min at the lowest setting of a Sorvall omnimixer homogenizer. The homogenate was then clarified by centrifugation at 3000 ***g*** and twice at 10,000 ***g*** for 10 min. The supernatant material was pelleted over 20% glycerol pH 7.8 at 98,000 ***g*** for 1 hr and the pellet was resuspended in 200 μl of 40 mM Tris–HCl.

RDDP activity was assayed by mixing 20 μl of the test sample with 20 μl of a solution containing 6 mM dithiothreitol and 0.4% triton X-100. After incubation for 15 min at 4°C, 20 μl of a reaction mix was then added to give a final volume of 60 μl, containing 40 mM Tris–HCl (pH 7.8), 60 mM KCl, 2 mM manganese acetate, 0.04 A260 units of either poly (dA): oligo (dT) or poly (rC): oligo (dG) and 4 μCi of either ^{3}H-thymidine triphosphate (*ca.* 40–60 Ci/mmole) or 4 μCi of ^{3}H-guanosine triphosphate (*ca.* 10 Ci/mmole).

The reaction solution was thoroughly mixed with the test sample and then 20 μl of the solution was spotted onto Whatmann filter paper (grade 541) to constitute the TO sample. Before use the filter papers were prewetted with 100 μl of 0.1M sodium pyrophosphate. The remaining solution was incubated for 60 min at 37°C and at the end of this period a further 20 μl aliquot was removed to form the T60 sample. When dry, the filter papers were washed with 10% TCA in 1% sodium pyrophosphate, followed by two washes in 5% TCA and a final wash in ethanol. The filter papers were prepared for scintillation counting, the background was subtracted and the results for each sample were recorded as a ratio of T60 counts/min:TO counts/min. Samples taken at TO and negative samples gave counts/min of about 400. A minimum positive for supernatant RDDP activity was taken as a T60/TO ratio of 4. In each assay a positive control sample of FeLV-A was included and this gave a T60/TO ratio of about 30.0. Apart from the subgroup A of FeLV, which is not permissive for canine cells [15], no other oncoviruses were employed in the laboratory.

^{3}H-Uridine labelling

Short term cultures of lymphosarcoma from case 21 were monitored for the production of RNA containing particles of oncoviral density by ^{3}H-uridine labelling followed by isopycnic density ultracentrifugation. Twenty ml cultures were labelled with 20 μCi/ml of ^{3}H-uridine (*ca.* 40 Ci/mmole). Radiochemicals, Amersham. After 24 hr the medium was removed, clarified and pelleted through 20% glycerol as described previously. The pellet was resuspended in 0.2 ml of 40 mM Tris–HCl and applied to a 3.8 ml, 15–65% sucrose gradient in TNE. Following centrifugation at 150,000 ***g*** for 3 hr, 3 drop fractions were collected from the bottom of the tube and 10 μl of each fraction were removed to determine the refractive index. The remainder of the fraction was washed onto glass fibre filters with TCA and the acid precipitable counts were measured in a scintillation counter.

RESULTS

Cell cultures

In short-term cultures of lymphosarcoma

cells not treated with halogenated pyrimidines there was usually a decline in the viable cell concentration for the first day. Proliferation of the cells followed so that, in most cultures, there was an increase in viable cell numbers above the original seeding density by the fifth day. In IDU and BrDU treated cultures the viable cell concentration on the fifth day of culture was more variable, lying between 2.5×10^5 and 1.2×10^6 viable cells/ml. However clumps of viable cells occurred within these cultures and the medium became acidic during the period of culture.

RDDP activity in culture supernatants

None of the culture supernatants from 14 cases of lymphosarcoma produced significant levels of polymerase activity with the poly (dA) : oligo (dT) template, whilst 3 of 14 cases had borderline levels of polymerase activity with the poly (rC) : oligo (dG) template (Table 1). Case 4 showed activity in the IDU treated supernatant with a T60/TO ratio of 5.1, whilst in case 6 activity occurred in the untreated culture with a T60/TO ratio of 4.8. Both the untreated and the IDU treated culture supernatants of case 8 possessed low levels of poly (rC) : oligo (dG) directed enzyme activity with T60/TO ratios of 4.3 and 4.7, respectively.

RDDP activity in tumour tissue

Tumour tissue from 11 cases of lymphosarcoma and two lymph nodes from clinically normal dogs were analysed for RDDP activity. Cases 8 and 21 showed definite activity with the poly (rC) : oligo (dG) template giving T60/TO ratios of 14.5 and 20.4, respectively (Table 1). No significant activity was detected with the poly (dA) : oligo (dT) template with these tissues.

^{3}H-Uridine labelled cultures

Two short-term cultures from case 21 were labelled with ^{3}H-uridine and the supernatants analysed by isopycnic density ultracentrifugation. Both cultures yielded radioactive peaks at a density of 1.15 g/ml (Fig. 1).

DISCUSSION

Evidence for the expression of retroviruses in canine lymphosarcoma has been limited to electron microscopic observations of oncovirus like particles [8, 9]. The experiments described here were a preliminary screening test for a retrovirus specific enzyme marker in canine lymphoid tumours.

In 3 of 14 culture supernatants, low, borderline levels of enzyme activity were demonstrable with a poly (rC) : oligo (dG) template. Although these supernatant assays require cautious interpretation, the enzyme preference for poly (rC) : oligo (dG) over poly (dA) : oligo (dT) and the lack of significant activity with the latter template would suggest that this activity was due neither to DNA-

Table 1. *Culture supernatant and tumour tissue RDDP activity assayed with poly (rC) : oligo (dG) and poly (rA) : oligo (dT) templates*

	Untreated		IDU/BrDU treated			Tumour tissue T60/TO	
	[dA : dT]	[rC:dG]	[dA : dT]	[rC : dG]	Case No.	[dA : dT]	[rC : dG]
1	1.1	2.8	N.D.	N.D.	4	2.8	0.4
2	1.4	2.0	N.D.	N.D.	8	2.4	14.5
3	1.3	2.0	1.8	2.5	15	1.7	4.9
4	1.6	3.5	1.5	5.1	16	2.8	2.8
5	1.4	1.2	N.D.	N.D.	17	2.0	3.8
6	1.9	4.8	1.7	3.0	18	1.7	5.0
7	1.0	1.0	0.9	1.1	19	3.1	3.0
8	1.3	4.3	1.2	4.7	20	2.3	2.1
9	2.4	2.3	1.9	3.2	21	1.4	20.4
10	3.6	2.1	3.8	1.9	22	2.1	3.4
11	1.7	2.7	1.3	2.7	23	2.8	4.0
12	2.7	2.2	N.D.	N.D.	A*	1.6	0.9
13	2.7	1.6	N.D.	N.D.	B*	1.7	1.1
14	N.D.	N.D.	1.3	1.4			

*Normal lymph nodes.
N.D. = not done.

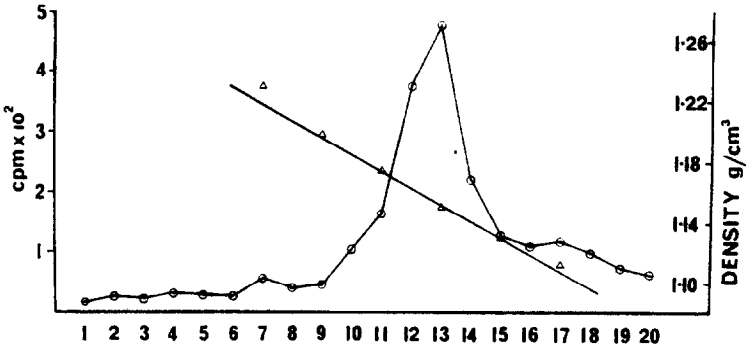

Fig. 1. An isopycnic density ultracentrifugation profile of a ^{3}H-uridine labelled culture of case 21. A radioactive peak was found at 1.15 g/cm^3.

DNA polymerases nor terminal transferase [16]. Apart from terminal transferase and some highly purified cellular enzymes, [17] polymerase activity with poly (rC) : oligo (dG) template is regarded as specific for RDDP activity of retroviruses [18]. The pattern of enzyme activity in the culture supernatants and the presence of activity in the tumour tissue preparations indicate that induction with halogenated pyrimidines was not necessary to detect limited expression of a poly (rC) : oligo (dG) directed enzyme.

In the tumour preparations, enzyme activity with poly (rC) : oligo (dG) in the two positive cases was considerably higher than in the tissue culture supernatants (Table 1). The crude preparations used in this assay are liable to contamination by cellular enzymes but the failure of these preparations to employ a poly (dA) : oligo (dT) template reduces the probability that terminal transferase or DNA–DNA polymerases were responsible for these results. Furthermore no poly (rC) : oligo (dG) directed enzyme activity was found in the two normal lymph nodes examined.

In both cases with positive enzyme activity in the tumour tissue limited confirmatory evidence was available for the presence of particles with oncoviral properties in the tissue culture supernatants. In case 8 this consisted of poly (rC) : oligo (dG) directed enzyme activity, whilst in case 21 there was evidence for RNA containing particles of oncoviral density in the tissue culture medium. Case 8 was an Old English Sheepdog bitch of 5 yr of age with a multicentric lymphosarcoma. This dog came from a household in which multicentric lymphosarcoma had been histologically confirmed in another Old English Sheepdog 4 months previously. These two dogs were not first degree relatives but had been present in the same house for several years. Case 21 was a 2-yr-old male Sheltie with a thymic lymphosarcoma which rosetted with the canine T-cell marker [19]. There were no other dogs present in the household and the litter mates were not traced.

In feline lymphosarcoma the tumours which contain the complete FeLV genome readily yield infectious virus and virus particles are numerous on electron microscopic examination of the tissue [20]. On the other hand, in bovine leukosis, virus is not easily demonstrable by electron microscopy of tumour tissue and expression of the virus may require a period of culture *in vitro* [21]. Similar mechanisms affecting the expression of leukaemogenic virus in the dog may be masking virus induced lymphosarcoma in this species and would account for failures to transmit this disease.

The data presented here are not sufficient to warrant the conclusion of retrovirus expression in the tumours examined particularly as no RDDP activity was found in the MDCK cocultures. Nevertheless the recent isolation of a retrovirus from a canine lymphosarcoma cell line (H. Strandström, in press) and the RDDP activity in the lymphosarcoma cases described here and by others (S. Armstrong *et al.*, personal communication) suggest that further investigation of a viral aetiology of these tumours should be undertaken. The occurrence of two cases of lymphosarcoma in the same household may also be of significance if the putative canine leukaemia virus is exogenous. Furthermore a common tumour associated antigen has been described for canine lymphosarcoma, a feature which would be concomitant with retrovirus expression in these tumours [22].

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