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Human Prostatic Reverse Transcriptase and RNA-Virus

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Summary. Methods for the assay of the RNA-dependent DNA polymerase which has the characteristics of the enzyme in murine leukemia virus have been adapted for study of extracts of human prostate in a search for RNA viral activity. Of the extracts from a total of 25 glands submitted to the evolved screening procedure 6 or 24% proved active enough to warrant further characterization, including tests of template dependence and specificity. In this set the extract from a carcinoma proved far more potent than any of the five coming from benign hyperplastic glands. It was not only dependent upon the presence of template but was a lot more responsive to the template most effective with virus, i.e., poly rA · oligo T (12 to 18) than with poly rA · poly T. Finally, the elution pattern of this extract's enzyme activity closely resembled that obtained from authentic viral extract. It was concluded from these preliminary experiences that this analytical scheme may be a simple means to investigate viral association with prostatic cancer.

Key words: Prostate cancer, RNA-virus, RNA-dependent, DNA-polymerase

Introduction

Evidence that cancer may be caused by viruses dates back at least to 1910 when Rous (9) showed the viral origin of sarcoma in chickens. Progress in extending these observations has been slowed by the technical difficulties of detecting these particles. For example, Bittner in 1940 (2) demonstrated that the tendency to develope breast cancer in HC strains of mice is transferred by the mother's milk. Yet it was 1963 when Dmochowski (3) found viral particles in the milk of animals with breast cancer, and 1964 when Lunger (6) et al. demonstrated such structures in milk fractions from normal and breast cancer patients. These findings inspired Tannenbaum et al. (10) to look for and indeed to find virions around cancerous areas in 48 of 61 human prostates studied with the electron microscope. Only in knowing the heroic effort which these early studies represent can one appreciate the revolutionary and simultaneous discovery by Baltimore (1) and of Temin and Mizutani (11) that RNA viruses contain an RNA-dependent DNA polymerase. This discovery puts in the hands of the biochemist an enzymatic test for readily screening tumors and tissues which may be tumor prone due to such viral infestation.

This report describes our preliminary experience in applying the reverse transcriptase assay to a series of extracts from benign hypertrophic and cancerous prostates.

Material and Methods

Human prostate samples were obtained from open surgical prostatectomies and transurethral resections. After setting aside representative bits of tissue for histological diagnosis and ultrastructural study, the remaining material was frozen or immediately extracted by the method of Evans (4) for reverse transcriptase assay:

Extraction. Homogenize two grams of fresh or freshly thawed prostate tissue in 10 ml of Tris-glycerol (0.5M Tris-HCL, pH 7.8, 0.001M manganese acetate, 0.5M KCL, 0.001M dithiothreitol (DTT), 20% glycerol). Add an equal volume of $(NH_4)_2SO_4$ solution (0.05M Tris-HCL, pH 7.8, 0.001M EDTA, $(NH_4)_2SO_4$ to saturation) to the homogenate. Mix and centrifuge 30 min at 30 000 X g. Discard supernate. Resuspend pellet in 2 ml Mn-free Tris-glycerol (0.02 Tris-HCL pH 7.8, 0.5M KCL, 0.001M DTT, 20% glycerol). Add Triton X-100 to 0.4% and incubate 15 min at 37°. Centrifuge at 100 000 X g for 1 hour. Either freeze or assay the resultant extract (supernatant fluid).

Reverse Transcriptase Assay adapted from Ross et al. (8) - Although other templates, e. g. poly rA · poly T, may be used as means for differentiating other enzymatic activity from that peculiar to RNA virus, the specific template for routine assay is poly rA · oligo T (12 to 18). The reaction mixture is composed of 5 parts 1M Tris-HCL, pH 8.3, 10 parts 0.02M DTT, 2 parts 3M NaCl, 1 part 1mM thymidine triphosphate (TTP), 10 parts ^{3}H-TTP, 500uCi/ml, 1 part 1mM poly rA, 1 part 0.1mM oligo dT (12 to 18), 1 part 0.1M $MnCl_2$, 5 parts 1% NP_{40}, 14 parts H_2O. Dilute an aliquot of viral or tissue extract with H_2O to 25 λ and mix in a small tube with 25 λ of the reaction mixture. Incubate 1 hour at 37°. Stop reaction by addition of 0.5ml, 0.02M $Na_2P_2O_7$ plus 2 drops of a suspension of 4 mg/ml yeast RNA. After mixing, add 0.05 ml 25% trichloroacetic acid (TCA) in saturated $Na_2P_2O_7$ and again mix. Wash suspension onto Millipore filter, 0.45u, which has been previously washed in saturated $Na_2P_2O_7$, with three rinses of cold 5% TCA. Dry filter 20 min in 100° oven and count in liquid scintillation counter.

Sephadex column (8). The column is packed with Sephadex G-100 in Mn-free Tris-glycerol. To establish the elution pattern of the reference virus, Murine Leukemia Virus (Moloney) obtained from Electro-Nucleonics Laboratories, Inc., Bethesda, Maryland, is solubilized in 0.5M KCL, 1.0M urea, 0.001M DTT, 1.0% Triton X-100 in 20% (v/v) glycerol and 150 λ applied to the column. The column is operated at a flow rate of around 50 ml/h. Effluent is concentrated 5:1 by dialysis through an Amicon concentrator. Each 20 minute fraction is assayed for reverse transcriptase activity.

Phosphocellulose chromatography (8). The phosphocellulose (Whatman P 11 Cellulose Phosphate) is washed first in 0.5N NaOH, then 0.5N HCL and adjusted to pH 6.5 with solid Tris. The sample is applied in 0.25M KCL and eluted with a linear gradient of KCL ranging from 0.25M to 0.8M. Fractions are assayed as usual.

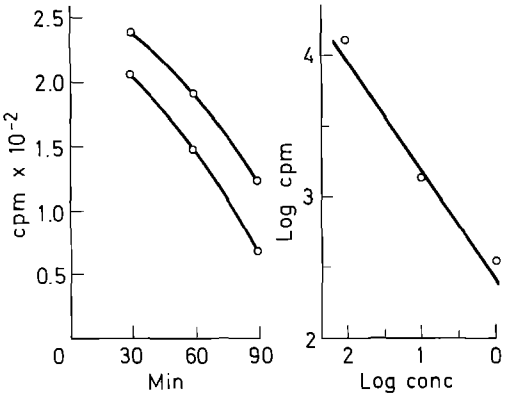

Fig. 1 Fig. 2

Fig. 1. Decay with time of enzymatic activity of 10 λ (upper curve) and 5 λ (lower curve) of viral extract shows as radioactivity in precipitated DNA after 30, 60 and 90 min incubation with substrate and poly rA · dT (12 to 18) template

Fig. 2. Dosage-response relationship of viral extract enzyme with standard assay conditions, the activity of 5, 0.5 and 0.05 λ of extract assayed

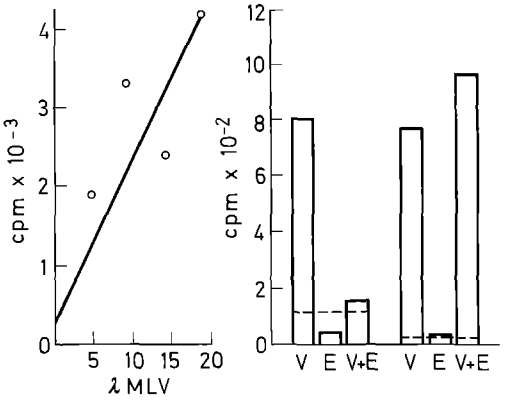

Fig. 3 Fig. 4

Fig. 3. More linear relation of enzyme activity to amount of viral extract in limited range of 5 λ to 20 λ

Fig. 4. Relative activities of viral enzyme prostate extract and their combination, using unfractionated extract in B, $(NH_4)_2SO_4$ - fractionated extract (8) in A. Horizontal line across each set of bars is blank value obtained without either viral or tissue extract

Results

It was necessary to determine the sensitivity and specificity of the assay on authentic virus before beginning study of tissue extracts. In early runs it was found (Fig. 1) that the yield of poly-DNA declined instead of increasing with time and was inversely related to amount of virus used. While not further investigated, these findings suggested that some DNAase activity is present in the viral preparation. We will see that either this or some polymerase inhibitor is also regularly found in prostatic tissue extracts (Table 1). Fig. 2 is a double logarithnic plot of virus concentration vs response. On this type of graph the inhibiting (?) effects of high virus concentrations are somewhat masked. The data from both of two experiments (of which this plot is a composite) showed maximal specific activity of the polymerase at the greatest dilution (least concentration). Fig. 3 shows quite a linear dosage-response relationship with the viral enzyme.

The very potent either inhibition of polymerase activity or DNA-depleting DNAase activity was

Table 1.

Patient	Diag.	Samp.Vol.	Cpm/ml	Prot/Ml ug	Cpm/Prot	β-Gluc units/mg	Cpm/β-g
1	AH+	10	6.890	2.562	2.68	35	1.97
2	AH+++	10	750	1.410	0.53	155	0.05
3	AH+	10	9.970	2.550	3.90	49	2.03
4	AH+ FMH++	5	23.320	3.250	7.17	88	1.32
5	AH+ PI++	10	8.070	3.490	2.31	136	0.59
6	ana. Ca	10	6.620	2.130	3.10	161	0.41
7	AH+++	10	1.400	2.120	0.65	48	0.03
8	AH+++	10	2.830	1.970	1.43	96	0.29
9	AH+++	5	9.170	2.340	1.16	150	0.61
10	AH+++	10	4.650	2.770	1.67	50	0.93
11	AH++	10	5.750	2.410	2.38	112	0.51
12	AH++	10	2.230	2.730	0.81	120	0.19
13	AH++	10	7.520	2.996	2.51	115	0.65
14	AH+++	10	4.110	1.680	2.44	-	-
15	AH+ FMH++	10	13.290	2.940	4.52	-	-
16	FMH+++ P+	10	0	-	-	-	-
17	AH+ FMH++	10	1.810	3.010	0.60	-	-
18	AH+++	10	8.910	2.069	4.30	-	-
19	Ca	10	16.800	-	-	-	-
20	FMH++	10	8.200	-	-	-	-
21	AH+	10	0	-	-	-	-
22	AH+ FMH+	10	0	-	-	-	-
23	AH+ I+	10	19.100	-	-	-	-
24	AH+++	10	7.400	-	-	-	-
25	AH+ FMH+	10	13.100	-	-	-	-

seen when extracts of Pt. 21 (Fig. 4) and Pt. 22 (Table 2) were mixed with viral extract. In general, the yield of DNA from viral enzyme is negated by some contribution from the tissue extract. A possible exception is seen in Figure 4: The extract used in A was obtained by $(NH_4)_2SO_4$ fractionation of the extract in B. In retrospect, I wonder if there was incomplete removal of the salt $(NH_4)_2SO_4$. Its presence could well explain the inhibition in A.

A time study with an extract from the prostate of Pt. 18 (Fig. 5) showed that best results are obtained with a 30 to 60 min incubation, just as with the defined viral extract.

Table 2. Effect of Tissue Extract (Pt. 22) on Viral Enzyme Activity

Experiment	Virus (V)	Tissue (T) cpm/vessel	V + T	Bkg
509	4251	493	549	467
510	3334	670	536	379
511	1384	677	958	216
512	520	344	384	506
$\bar{X}$	2372	546	607	392

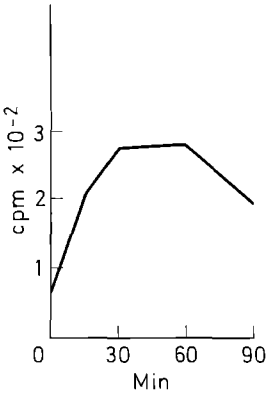

Fig. 5. Effect of time of incubation of a tissue extract on yield of poly DNA

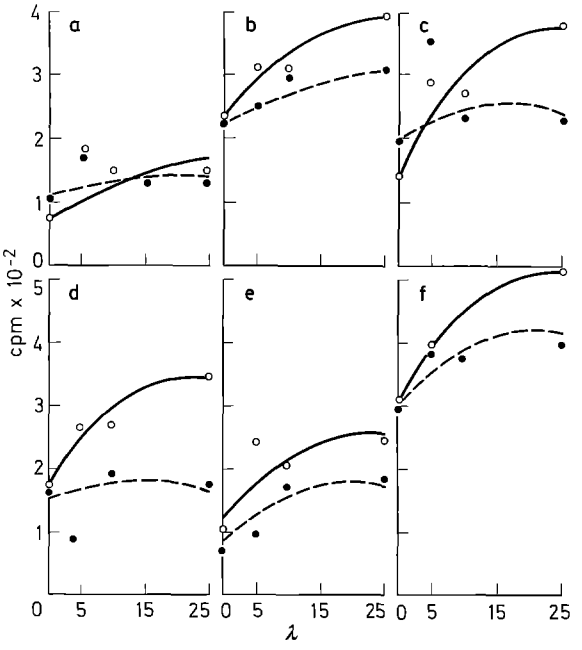

Fig. 6a-f) Results of assays of 5 λ to 25 λ aliquots of extracts from 6 patients with (0 - 0) and without (+---+) poly rA · oligo T (12 to 18). Patients b-e, 24, 25, 20 and 13, respectively had benign hypertrophic prostates; a, Pt. 6, and f, Pt. 19, had anaplastic and well differentiated, extensively metastasized prostate cancer

In Fig. 6 extracts prepared from prostates of 6 patients are compared. Three different sized aliquots of each were assayed both with and without the poly rA · oligo dT template. It can be seen that in general the activity of all tissue and extracts were about equal and low without the template. More important to the object of this study, one notes how much more active is the extract of Pt. 19 (Fig. 6f) (the only patient in the group with differentiated metastatic carcinoma) than those from the five other patients who had benign prostatic hypertrophy (Fig. 6 a-e). The magnitude of polymerase activity shown by the Pt. 19 extract (Fig. 7) with the poly rA · poly T template indicated that this preparation is diluted with non-specific (probably not viral) activity. Hopefully, the difference between the two rates may be a measure of specific viral enzyme activity in this extract.

Table 2 summarizes assay data on extracts from 25 patients' prostates. The non-linear dosage-response of most extracts, alluded to earlier, is seen in assay data on several aliquots. The biuret reaction (7) was used to measure the protein concentration of most extracts with significant activity. Some of the more recently acquired prostate specimens were tested for β -glucuronidase activity (5), too, in an effort to determine the correlation of reverse transcriptase with the epithelial content of the tissues.

A composite of the elution patterns of four extracts of the virus is shown in Fig. 8. The principal peak emerged at nearly the same point as that of Ross et al. (8) (Cf figure 9, redrawn from Ross's data to our scale). Our viral extracts regularly had a second peak of activity at around 50 to 55 ml post-void volume. Sephadex G-100 elution patterns of extracts from 4 glands of relatively high specific transcriptase activity are shown in Figure 10. Patient 1's plot (Fig. 10a) is most nearly confluent with that of the viral ex-

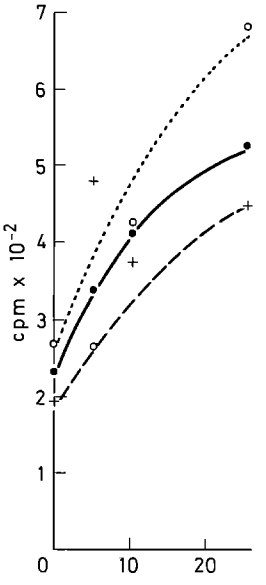

Fig. 7. Demonstration of greater activity of Pt. 19 extract with poly rA · oligo T (12 to 18) than with poly rA · poly T (+ - - +) and effect of concentrating the extract (O ... O) on response with poly rA · oligo T (12 to 18) template

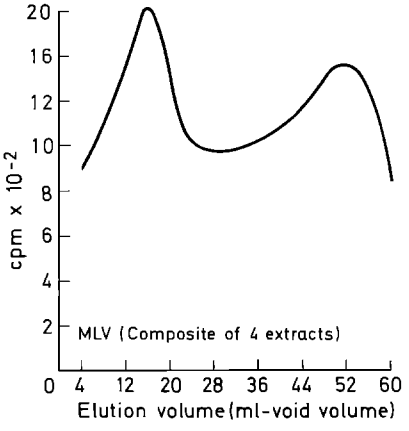

Fig. 8. Composite of elution patterns of 4 extracts of the virus separately chromotrographed on Sephadex G-100 column

tract. A principal peak at 48 ml instead of at 20 ml distinguishes Pts. 9 (Fig. 10b), 3 (Fig. 10c) and perhaps 4 (Fig. 10d). Our earlier extensive testing of the Pt. 19 extract left none for column analysis.

Finally, a phosphocellulose column was calibrated with virus extract (Fig. 11, solid line) and used for chromatography of extracts of Pt. 9 (AH +++) (dashed line) and Pt. 6 (poorly differentiated Ca) (short dashed line). The former of these two had a good peak of activity. However, it emerged

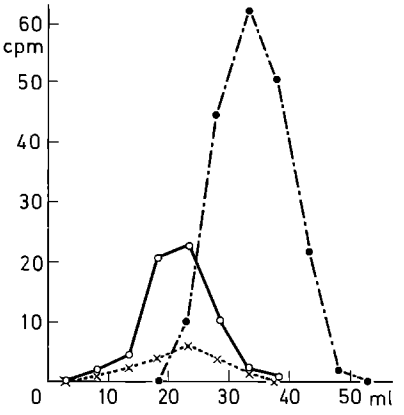

Fig. 9. Elution pattern obtained by Ross with viral extract on Sephadex. His data have been replotted to match scale and format of this study

far earlier than that from the virus. No significant activity was detectable in the extract of the cancer patient, Pt. 6, perhaps due to the extensive dilution in chromatography of an already low specific activity extract.

Discussion

While the extent of this study is inadequate to produce definitive answers to the question of RNA virus infestation and/or action in prostatic cancer, it does show that such analyses are possible; that viral activity may well be present. If so, it is not peculiar to the diagnosed cancer. Activity was most vigorous in the cancer patient, Pt. 19, but was also prominent in the BPH extract of Pt. 1 Even the elution patterns from the Sephadex column were characteristic.

We believe the hint of viral reverse transcriptase activity in tissue not diagnosed as cancerous does not invalidate the study; that it may either forecast cancer or the potential for such development. Such findings are compatible with both the protovirus and the oncogene hypotheses (12). The first of these holds that virions per se do not pre-exist but rather arise from protoviruses - genetic systems which transmit information from DNA through RNA to new DNA. Thus if a free DNA form of a protovirus happens to integrate with a region of the genome which could form membrane protein, the genome of an RNA virus would result. The oncogene idea is that the organism carries repressed viral genetic material within the genome which can, under appropriate conditions, be derepressed (turned on). Perhaps an inappropriate hormonal environment or circumstance such as described by Warburg (13) could trigger the turning on.

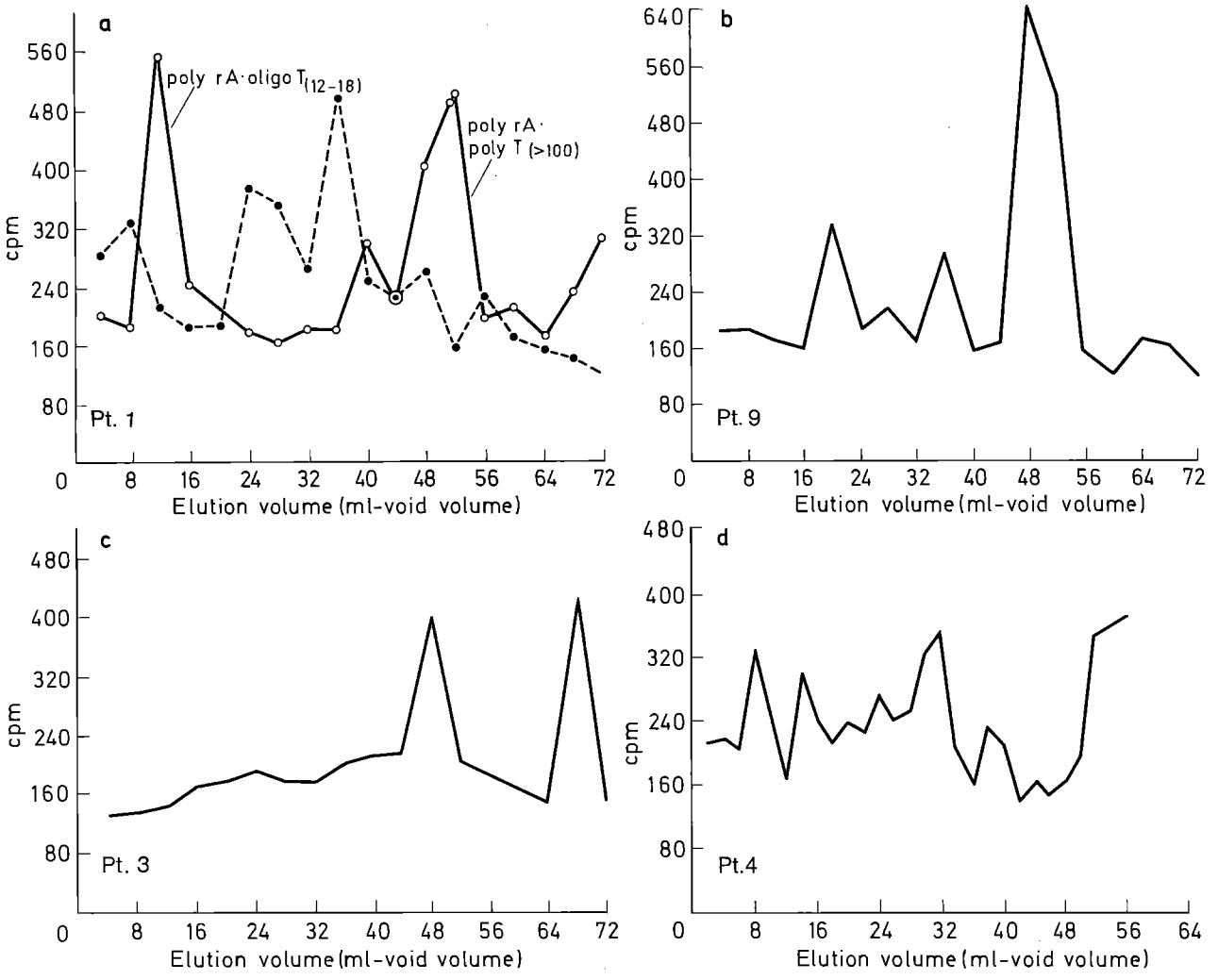

Fig. 10a-d) Sephadex elution patterns of 4 patients

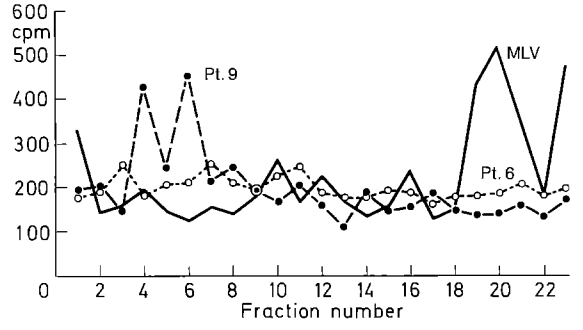

Fig. 11. Elution patterns of virus extract (———), Pt. 9 (benign hypertrophy) (---) and Pt. 6 (poorly differentiated CA) (cf Figure 6A) (-----)

If our arguments are valid, we recommend the above screening program both for investigating the viral etiology of prostatic cancer and for early detection of the imminent or emerging disease.

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