OCCURRENCE OF PARTICLE-BOUND REVERSE TRANSCRIPTASE IN HUMAN AMNIOTIC FLUID

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SUMMARY: Reverse transcriptase-like activity associated with particles banding at densities characteristic of RNA tumour viruses has been isolated from human amniotic fluid obtained from 16-22 weeks old pregnancies. It has been purified 2,000 fold through several sucrose sedimentation gradients. Like viral related reverse transcriptase, it utilizes Oligo (dG).poly(C) and heteropolymeric mRNAs as template-primers. Under optimised assay condition for different template-primers tested it favours Oligo (dG).poly(C) over others. It was only slightly inhibited by antiserum to reverse transcriptase from gibbon ape type-C virus.

Detection of reverse transcriptase in human leukemic cells (1,2) and human breast cancerous tissues (3) suggests the presence of viral information in affected human tissues. Reverse transcriptase has also been detected in normal embryonic tissues of different species of animals (4-7). The only report on human tissue relates to the study using tissue culture cells by Panem et al. (8) which described the presence of reverse transcriptase in type C viruses released from human embryonic fibroblasts after long term culture. Findings relating to the presence of reverse transcriptase in normal cell have led to the hypothesis (9) that this enzyme may have a role in normal cell physiology.

The present study describes the presence of reverse transcriptase in fresh (uncultured) human amniotic fluid of second trimerter pregnancy.

MATERIALS AND METHODS

$[^3H]$ dTTP (16 Ci/mmole); $[^3H]$ dGTP (14 Ci/mmole) were obtained from New England Nuclear Corporation, Boston, Massachusetts. Oligo (dT).poly(A); Oligo (dT).poly(dA); Oligo (dG).poly(C) and Oligo (dG) were obtained from Collaborative Research, Waltham, Mass. Activated Salmon Sperm DNA was prepared following the method of Schlabach et al. (10). Messenger RNAs were prepared from whole human fetal tissue by the method described earlier (11). Embryonic tissues were obtained from K.E.M. Hospital, India. Gibbon ape viral reverse transcriptase and antiserum (IgG, Immune gamma globulin) to this enzyme were kindly provided by Dr. R.C.Gallo of National Cancer Institute, Bethesda, Md.

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Table 1. Recovery of Oligo (dG).poly(C) directed enzyme activity during purification.

Purification steps	Total protein (mg/fraction)	Total nucleotide incorporated (pmole/fraction)	Purification	
			Sp. activity (pmole/mg.prot)	Purification factor
Fr. I	80.0	1.40	0.017	1
Fr. II	25.0	8.50	0.340	20
Fr. III	12.0	42.00	3.500	205
Fr. IV	3.8	155.0	40.740	2398

The purification steps were described in materials and methods. DNA polymerase assays were done as described in experimental section using Oligo (dG).poly(C) template-primer. 20 µl aliquots from each fraction were assayed for enzyme activity at 37°C for 1 h.

Isolation of enzymes

Early human embryos with amniotic sac intact were collected shortly after hysterotomy of 16-22 weeks old unwanted pregnancies. The amniotic fluid was collected asceptically by a syringe and then the fetus was separated from amnion and placental tissue. The fluid thus collected was processed immediately or could be stored at -20°C for 1 month. Storage under these conditions did not cause substantial loss of enzyme activity. It was centrifuged at 12,000 x g for 10 min and the supernatant obtained was centrifuged at 100,000 x g for 1 h in order to obtain the microsomal pellet. The pellet (Fr. I) thus obtained was suspended in TNE buffer (Tris-HCl at pH 8.0, 10 mM; EDTA, 0.1 mM; NaCl, 0.1 M), layered on a discontinuous sucrose gradient in TNE buffer, containing 20 and 50% sucrose (W/W) and finally subjected to centrifugation at 100,000 x g for 2.5 h. Active fraction (Fr. II) that rested on 50% sucrose layer was monitored by Oligo (dG).poly(C) templated reaction. It was carefully collected and diluted with TNE buffer and placed on 20-35% (W/W) linear sucrose gradient and finally centrifuged at 100,000 x g for 1 h. The active enzyme fractions (generally the upper part of the gradient were pooled and pelleted (Fr. III) at 100,000 x g for 1 h. Fr. III was layered on 20-60% (W/W) linear sucrose gradient in TNE buffer and run for 13 h at 100,000 x g. The gradient was fractionated from the bottom. The active fractions monitored by Oligo (dG).poly(C) templated reaction, were pooled and dialysed against buffer B (Tris-HCl at pH 8.0, 10 mM, dithiothreitol 10 mM; phenylmethyl sulphonyl fluoride, 0.01 mM; glycerol, 10%). The enzyme preparation thus obtained (Fr. IV) could be stored with bovine serum albumin (10 mg/ml, F.C.) for 2-3 months in liquid nitrogen.

DNA polymerase assay

A standard reaction mixture (0.1 ml) contained 50 mM Tris-HCl at pH 8.0; 10 mM dithiothreitol; 0.1 mg/ml bovine serum albumin; 0.1% Nonidet p-40; 1 mM ATP; 16 uM [^{3}H] dNTPs corresponding to relevant synthetic template primer

Table 2. Frequency of the presence of reverse transcriptase in amniotic fluid at gestational ages.

Case	Fluid content (in ml)	Gestational age (in weeks)	Total dGMP incorporated (pmole/10 ml fluid)
I	198	18	7.50
II	182	16	7.00
III	210	22	6.25
IV	193	19	0.00
V	222	20	5.90
VI	415	36	0.00
VII	393	38	0.00

Amniotic fluid was processed separately for enzyme isolation following the method described in experimental section. Samples were processed upto isopycnic sucrose density gradient purification step before it was declared negative. DNA polymerase assays were done as described in Table 1.

which were used at 0.1 mg/ml; 0.5 mM $MnCl_2$; and 80 mM KCl. In case of mRNAs; endogenous template; and activated DNA templated reaction 75 uM of each unlabelled dATP, dCTP, dTTP and 16 uM of [^{3}H] dGTP were used. [^{3}H] dGTP, 4,123 cpm/pmole or [^{3}H] dTTP, 6770 cpm/pmole were used wherever necessary. All reactions were incubated at 37°C for 1 h unless otherwise stated and were stopped by 10% TCA containing 0.02 M sodium pyrophosphate and acid precipitable cpm was counted in toluene fluor, after it was washed with 5% TCA containing 0.02 M sodium pyrophosphate. Acid precipitable cpm in each enzyme assay was corrected for background cpm, obtained with an identical reaction conducted by heat denatured enzyme which ranged between 500 - 1,000.

Protein estimation

Protein was estimated by the method of Lowry et al. (16).

RESULTS

Isolation and purification of particle-bound reverse transcriptase

It is evident from Table 1, that particulate fraction (Fr. I) obtained from amniotic fluid had detectable amount of reverse transcriptase activity (0.017 pmole/mg of protein). Purification through several steps (Fr. II, III, and IV) enriched the activity to 40.74 pmole/mg. protein. This shows at least

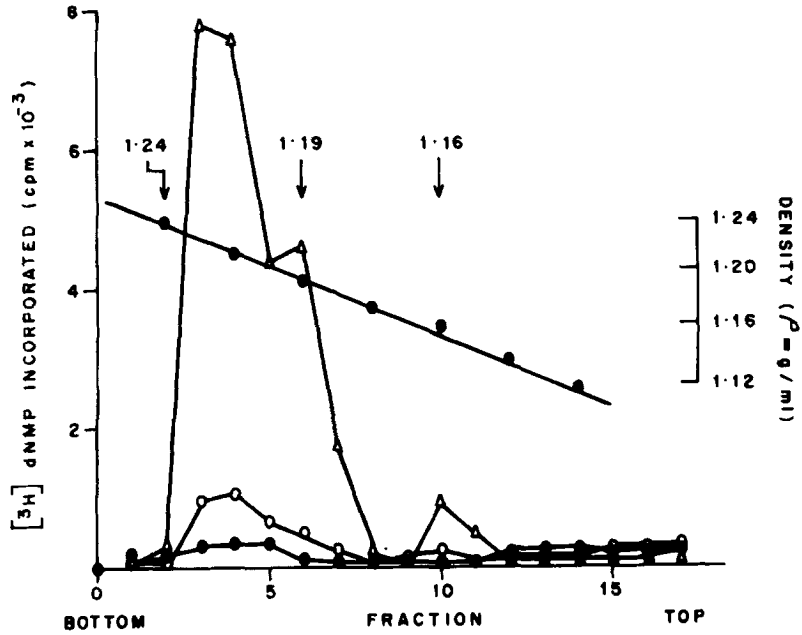

Figure 1. Isopycnic sucrose gradient analysis of Fr. III preparation. After specified run, the gradients were fractionated into 17 equal parts. Fractions were collected from bottom by siphon action. DNA polymerase activity in each fraction was monitored by deoxyribonucleotides incorporation in response to different template-primers. The assay procedure was described in Materials and Methods. 20 µl aliquot of each fraction was used for DNA polymerase assay. DNA polymerase activity in response to:Oligo (dG).poly(C), (Δ—Δ); Oligo (dT).poly(A), (O--O); Oligo (dT).poly(dA) in Mn^{2+}, (●-●).

2,000 fold purification of activity as expressed in pmole dGMP incorporated/mg. protein.

Reverse Transcriptase in amniotic fluid of different gestational ages

It is seen from Table 2 that amniotic fluid obtained from 4 out of 5 cases of second trimester pregnancies showed considerable dGMP incorporating activity whereas it was not possible to detect any activity in the fluid of term pregnancies.

Density of the particles

Fig. 1 shows that the particles associated with enzyme activity band at densities of 1.24 - 1.17 g/ml which are also characteristic of RNA tumour

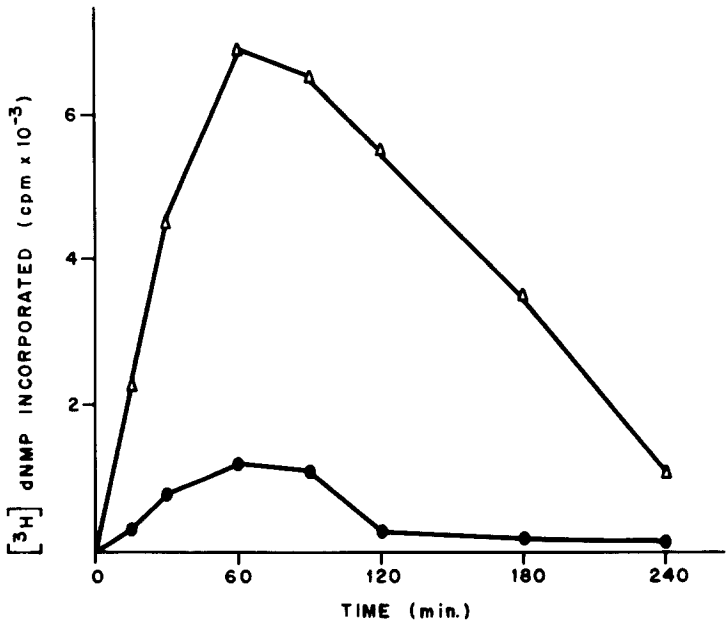

Figure 2. Kinetics of DNA synthesis. DNA polymerase activities were assayed as described in the experimental section with 20 µl aliquot of Fr. IV enzyme preparation. DNA polymerase activity in response to: Oligo (dG).poly(C), (Δ–Δ); Oligo (dT).poly(A), (●–●).

viruses. It may be noticed in Fig. 1 that major part of particles carrying enzyme activity banded at 1.22 g/ml which is typical of type C viral core as well as intact type B virus. The minor peak at 1.17 g/ml corresponds to intact type C virus. It is also evident from Fig. 1 that Fr. III does not show any other DNA polymerase activity.

Kinetics of transcription

As the partially purified Fr. IV is highly contaminated with other proteins which may interfere with the assay procedure, attempts were made to optimize the conditions for the DNA polymerase assay in relation to time. It is seen in Fig. 2 that within an hour the enzyme activity attained its peak and then declined. This is found with two different template-primers.

Utilization of template-primers

Preference of the enzyme for various templatesare shown in Table 3.

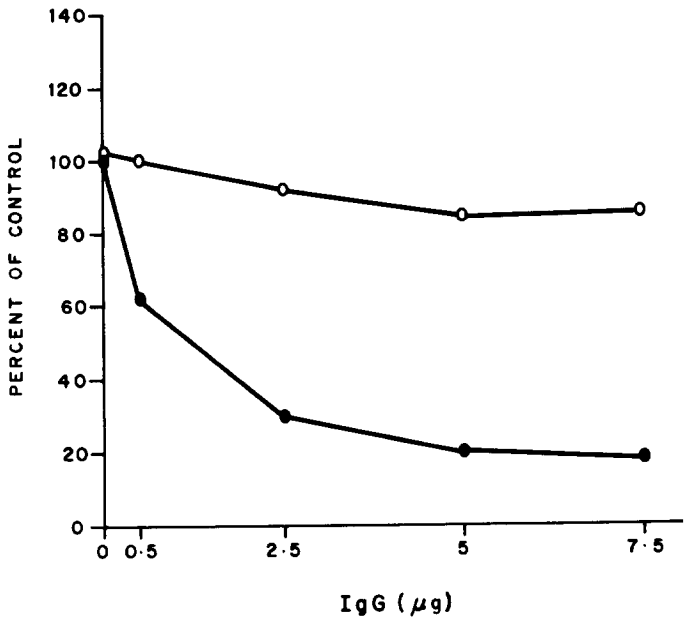

Figure 3. Effect of antisera (IgG) to reverse transcriptase from gibbon ape type C virus on reverse transcriptase from human amniotic fluid and from gibbon ape virus. 20 µl aliquot of enzymes in buffer B was preincubated at 4°C for 1 hr. with 10 µl of immune gamma globuline preparation of different protein strength in the presence of 5 µl of 2% NP-40 and 5 µl of 1.6 M KCl. Assays for the incorporation of (^{3}H) dGMP on Oligo (dG).poly(C) were performed as described in the experimental section. From 6,000 to 8,000 cpm in case of human and 14,000 to 16,000 cpm in case of viral enzyme were observed in the absence of antisera. The values were expressed as percent of activity in the absence of antisera. Gibbon-ape viral enzyme,(●--●); Human amniotic enzyme,(O-O).

The enzyme preparation (Fr. IV) from amniotic fluid reveals the following template preference: Oligo (dG).poly(C)>Oligo (dT).poly(A)>Oligo (dT).poly (dA)> mRNAs >Endogenous templates. The absence of enzyme activity when Oligo (dG) was used without a primer eliminates the possible presence of deoxyterminal transferase in this preparation. Utilization of natural mRNA as template with an initiator Oligo (dT) suggests that this activity is due to reverse transcriptase. It is also seen that this preparation can utilise the endogenous template, although with less efficiency. As the addition of RNAase inhibited the polymerization in presence of endogenous template, it is apparent that the endogenous template may be an RNA.

Table 3. Some features of template-primer utilization by amniotic enzyme.

Template-primer	Assay condition	Sp. activity (pmole/mg.prot.)
Oligo (dG).poly(C)	Complete	40.74
Oligo (dG)	"	0.00
Oligo (dT).poly(A)	"	8.12
Oligo (dT).poly(dA)	Complete	6.00
"	- Mn^{2+} + Mg^{2+}	4.00
"	- 70 mM KCl	3.00
Activated DNA	Complete	0.00
mRNAs + Oligo (dT)	Complete	5.10
"	- Oligo (dT)	0.40
"	- dATP	0.00
Endogenous template	Complete	0.80
"	+ RNAase A (10 µg)	0.00

Reaction mixtures for complete assay systems with respect to different template-primers were described in Materials and Methods. [^{3}H] dTTP was used as substrate in case of Oligo (dT).poly(A) and Oligo (dT).poly(dA) templated reaction, otherwise [^{3}H]dGTP was used. In case of endogenous template, activated DNA and mRNAs templated reactions, three other nucleotides were added in addition to [^{3}H] dGTP. In these cases pmole value of substrate incorporation was calculated by multiplying the dGTP value by 3.5. Following template-primers were used in certain assays as indicated in the Table: mRNAs, 7.8 µg; Oligo (dT), 2 µg; activated DNA, 10 µg. The concentration of endogenous template, whichever was present in 20 µl of Fr. IV enzyme preparation, was not determined. Actinomycin D (50 µg/ml) was used for endogenous template and messenger RNA templated reactions. 20 µl aliquots of Fr. IV enzyme were used for each assay. Mean value of three such experiments were documented in Table 3.

Immunological properties of the enzyme

Fig. 3 shows that antiserum (immunogamma globulin) to reverse transcriptase of gibbon ape leukemia virus inhibited human amniotic reverse transcriptase by only 17%, whereas the same concentration of the antiserum inhibited gibbon ape viral reverse transcriptase by 80% when tested under identical assay conditions. This indicates that there is only a feeble immunological cross-reactivity between the enzyme isolated from human amniotic fluid and gibbon ape viral reverse transcriptase.

DISCUSSION

Selective preference to different template-primers is one of the criteria, distinguishing the various forms of DNA polymerases and also reverse transcriptase. The template Oligo (dT).poly(A) which earlier was thought to be specific for reverse transcriptase has now been shown (11-14) to be utilized also by β & γ DNA polymerases. However, Oligo (dG).poly(C) or mRNAs are specifically utilized by reverse transcriptase. The evidence given here shows that the enzyme isolated from human amniotic fluid conforms to the criteria of reverse transcriptase (See Table 3) as based on the preference shown to various template-primers. The association of this enzyme with particles of densities 1.24 - 1.17 gm/ml characteristic of RNA tumour viruses suggests the possible presence of RNA viral information, in human amniotic fluid as has also been reported in reverse transcriptase prepared from chicken amniotic fluid (5). Human amniotic reverse transcriptase is more active with Oligo (dG).poly(C) than to Oligo (dT).poly(A). In contrast, human leukemic cell reverse transcriptase (1,15) prefers Oligo (dT).poly(A) to Oligo (dG).poly(C). Unlike human leukemic reverse transcriptase the enzyme isolated from human amniotic fluid is very marginally inhibited by antisera against gibbon ape viral reverse transcriptase (Fig. 3). Thus it appears that human amniotic reverse transcriptase is not identical with the reverse transcriptase isolated from human leukemic cells (1,2). The data given here demonstrates the presence of reverse transcriptase activity in the amniotic fluid of 80% cases examined. Thus it may be presumed that it is not the result of cross contamination by exogenous RNA viruses. It is of interest that it was not possible to demonstrate the presence of reverse transcriptase activity in amniotic fluid of term pregnancies and also in other fetal tissues at different gestational ages.

Occurrence of particle bound reverse transcriptase in human amniotic fluid seems to suggest that this enzyme has a function in normal cell physiology. It may also indicate that the latent endogenous RNA viral information is expressed only in certain stages of embryonic development.

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