RELATEDNESS OF RNA AND REVERSE TRANSCRIPTASE FROM HUMAN ACUTE MYELOGENOUS LEUKEMIA CELLS AND FROM RNA TUMOR VIRUSES

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Abstract

Complexes of RNA tumor virus-like RNA (35S or 70S RNA) and DNA product, synthesized endogenously from deoxynucleotide triphosphates by a particulate cytoplasmic fraction, were detected in leukemic cells from seven of eight cases of human acute myelogenous leukemia. In one case, the DNA reaction product was shown to have extensive sequence homology to RNA from a primate C-type virus. In two other cases, a RNA-directed DNA polymerase with biochemical and immunological relatedness to reverse transcriptase from primate C-type viruses was isolated from the endogenous, DNA-synthesizing cytoplasmic fraction. Thus, human leukemic cells contain virus-related RNA and RNA-directed DNA polymerase (reverse transcriptase), which are associated in a fashion similar to analogous molecules in RNA tumor viruses. These findings suggest that C-type viruses are etiologically related to leukemia in man, as in lower species, and that immunobiochemical products from primate C-type viruses may provide sensitive probes for further exploring the role of viral information in human leukemia. They also substantiate our earlier reports on reverse transcriptase in human leukemic cells.

Introduction

In the past two years evidence has accumulated that human leukemic cells contain molecules with marked similarity to analogous components from RNA tumor viruses (1–7). One experimental approach, followed by Gallo and his associates, has resulted in the identification and purification from both acute lymphoclastic and acute myeloblastic leukemia cells of RNA-directed DNA polymerases, which use synthetic template-primers and natural RNA-primers in a similar fashion to purified reverse transcriptase from RNA tumor viruses (1, 2, 7). Recent evidence further indicates that leukemic RNA-directed DNA polymerase is inhibited by antisera to reverse transcriptase from some mammalian C-type viruses (ref. 3 and *vide infra*). A second experimental approach, advanced by Spiegelman and his colleagues, has accomplished the identification in acute and chronic myelogenous and lymphatic leukemia cells of cytoplasmic RNA's, which has small but detectable sequence homology with RNA

from a mouse type-C leukemia virus (Rauscher) but not with RNA from avian leukemia virus or from mouse mammary tumor virus (5, 6). Recently, our laboratory has found more extensive homology of RNA from human acute leukemic cells to primate C-type sarcoma virus RNA (ref. 4 and *vide infra*). Both experimental approaches indicate that the RNA tumor virus-like polymerase and RNA are complexed, as determined by the endogenous synthesis of DNA on the RNA template (2–5), and that the polymerase-RNA complex is present in a cytoplasmic particle which has the density (1.14–1.17 g/ml) characteristic of RNA tumor viruses (3–5). The latter suggests that some virus-like form exists in leukemic cells, however, no discrete morphological entity or infectious particulate activity has yet been identified. Also, both experimental approaches have failed to find RNA tumor virus-like molecules in normal leukocytes, although this could be due to inadequate sensitivity of the techniques or to the unavailability of completely appropriate control cells, i. e. normal myeloblastic or lymphoblastic stem cells.

In the present report, we summarize some of our recent findings supporting the presence of RNA tumor virus-like RNA and RNA-directed DNA polymerase in human acute myelogenous leukemic cells. These data are related to results obtained by velocity glycerol gradient analyses of endogenous DNA reaction products from a particulate cytoplasmic fraction of leukemic cells from eight cases of acute myelogenous leukemia. The preparation of the cytoplasmic pellet fraction was modified from a previously reported method (2). The velocity gradient assay for the "simultaneous detection" of RNA template and associated DNA polymerase is a slight modification of the previously described method (8).

Fig. 1: Velocity glycerol gradient analysis of endogenous ^{3}H-DNA product synthesized by the cytoplasmic "pellet" fraction of acute myelogenous leukemia cells (P. C.). The cells (0.75 gm), suspended in 5 volumes of buffer A (0.05 M Tris, 5pH 7.5, 5 mM $MgCl_2$, 0.02 M dithiothreitol and 0.5 mM EDTA) containing 0.1 m sucrose, were homogenized to approximately 75 % cytoplasmic rupture in a tightfitting Dounce homogenizer. The nuclei and mitochondria were successively removed by centrifugation for 15 min. at 1000 xg and 12,000 xg, respectively. The supernatant was layered over a 10 ml column of buffer A containing 25 % (w/w) sucrose in an SW27 rotor and centrifuged at 98,000 xg for 1 hour. The supernatant was removed and the "cytoplasmic pellet fraction", was evenly suspended in 0.5 ml buffer B (0.05 M Tris, pH 7.5, 5 mM $MgCl_2$, 0.1 M KCl, 1 mM dithiothreitol and 0.5 mM EDTA). Triton X-100 was added to a final concentration of 0.1 %, and the suspension was incubated at 4 °C for 15 min. In a final volume of 1.25 ml containing 0.25 ml of the pre-incubated pellet, 50 μg/ml actinomycin D, 0.05 M Tris, ph 8.3, 60 mM KCl, 15 mM KF, 10 mM $MgCl_2$, 5 mM ATP, 320 μM dGTP and approximately 30 μM each ^{3}H-TTP (Amersham, 54 Ci/mMole), ^{3}H-dATP (Schwarz, 12.5 Ci/mMole) and ^{3}H-dCTP (Schwarz; 23 Ci/mMole), DNA synthesis was performed at 27 °C for 30 min. The reaction was terminated and the DNA product purified as described by Reitz, et al. (9). The purified DNA product was applied to a 10–30 % gradient of glycerol in TNE buffer (0.01 M Tris, pH 7.5, 0.1 NaCl and 1 mM EDTA) and centrifuged in a SW41 rotor at 40,000 RPM for 4 hr. Samples were collected and processed as previously described (9). An external marker of ^{3}H-70S RNA from avian myeloblastosis virus was included in the centrifuge run. ●——●, untreated DNA product; ○——○, DNA product treated with RNase A (Worthington), 20 μg/ml, at 37 °C for 30 min.

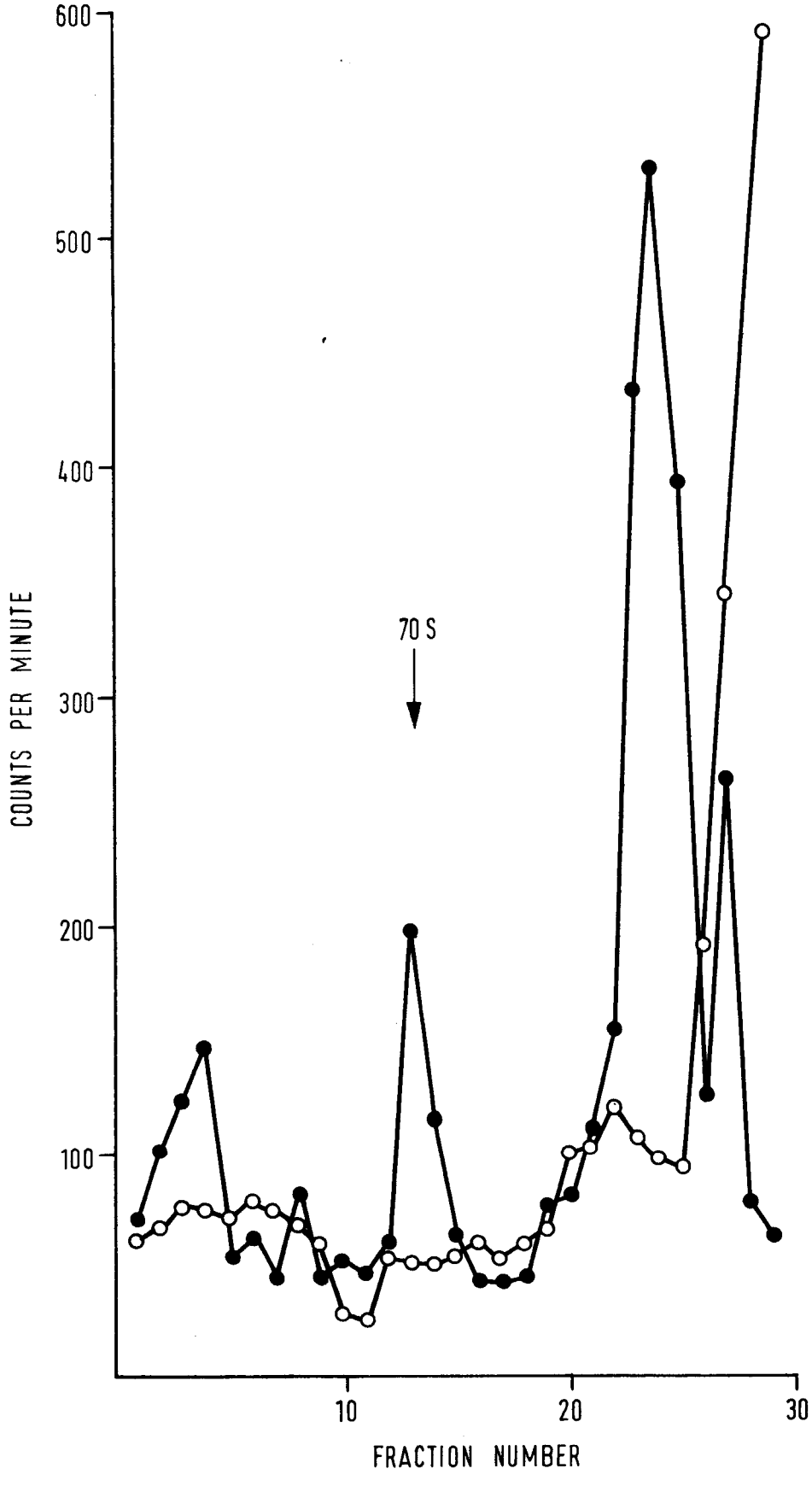
600
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Fig. 1

Methods and Results

Myelogenous leukemic cells used in these studies were obtained by plasmaphoresis from patients with the clinical diagnoses of acute myelogenous leukemia (AML), acute myelomonocytic leukemia (AMML) and chronic myelogenous leukemia in the acute, blastic phase of the disease (CML). The cytoplasmic pellet fraction of the leukemic cells was prepared by first removing the nuclei and mitochondria from homogenized cells by differential centrifugation and by then further centrifuging the post-mitochondrial supernatant fraction at 98,000 x g for 1 hour. The material thus "pelleted" was used for the endogenous synthesis of DNA from four added deoxynucleotide triphosphates (see the legend to Figure 1 for details) and for further purification procedures described in the text.

Velocity Glycerol Gradient Analyses of Endogenous DNA Product

As illustrated in Figure 1 and summarized in Table 1, ^{3}H-DNA product associated with a rapidly-sedimenting, ribonuclease-sensitive complex was detected in 4 of 8 cases in which fresh or fresh-frozen (−70° C in 10 % dimethylsulfoxide) myelogenous leukemia cells were studied. The rapidly sedimenting ^{3}H-DNA-RNA complex was not observed if one deoxynucleotide triphospate was omitted from the endogenous DNA synthesizing reaction mixture (Table 1; Figure 2A, 3B) indicating that template-independent, end-addition reactions were not being detected (5). In all cases, the ^{3}H-DNA-RNA complex sedimented at the location of ^{3}H-70S and ^{3}H-35S

Table 1. Velocity gradient analyses of ^{3}H-DNA products from the 98,000 xg cytoplasmic pellet fraction of human leukemic blood cells*

		Rapidly-Sedimenting (RS) DNA				
Patient	Diagnosis	Estimated Size	cpm RS DNA	Per cent Total cpm	RNase Sensitivity	−1 dXTP (% of cpm)
P.C.	AML‡	70S	495	7.8	+	N.D.+
N.N.	AML		N.D.			
G.F.	AML	35S	1830	50.4	+	7.6
J.C.	AMML§		N.D.			
C.P.	AMML	35S	110	10.7	+	N.D.
J.Wh.	CML¶		N.D.			
J.Wi.	CML		N.D.			
A.W.	CML	35S	95	2.1	+	N.D.

* Procedures used for the preparation of the cytoplasmic pellet fraction, for the synthesis and purification of ^{3}H-DNA product, and for velocity glycerol gradient analysis are described in Figure 1.

\+ N. D., not detected.

‡ AML, acute myelogenous leukemia

§ AMML, acute myelomonocytic leukemia

¶ CML, chronic myelogenic leukemia in blast crisis.

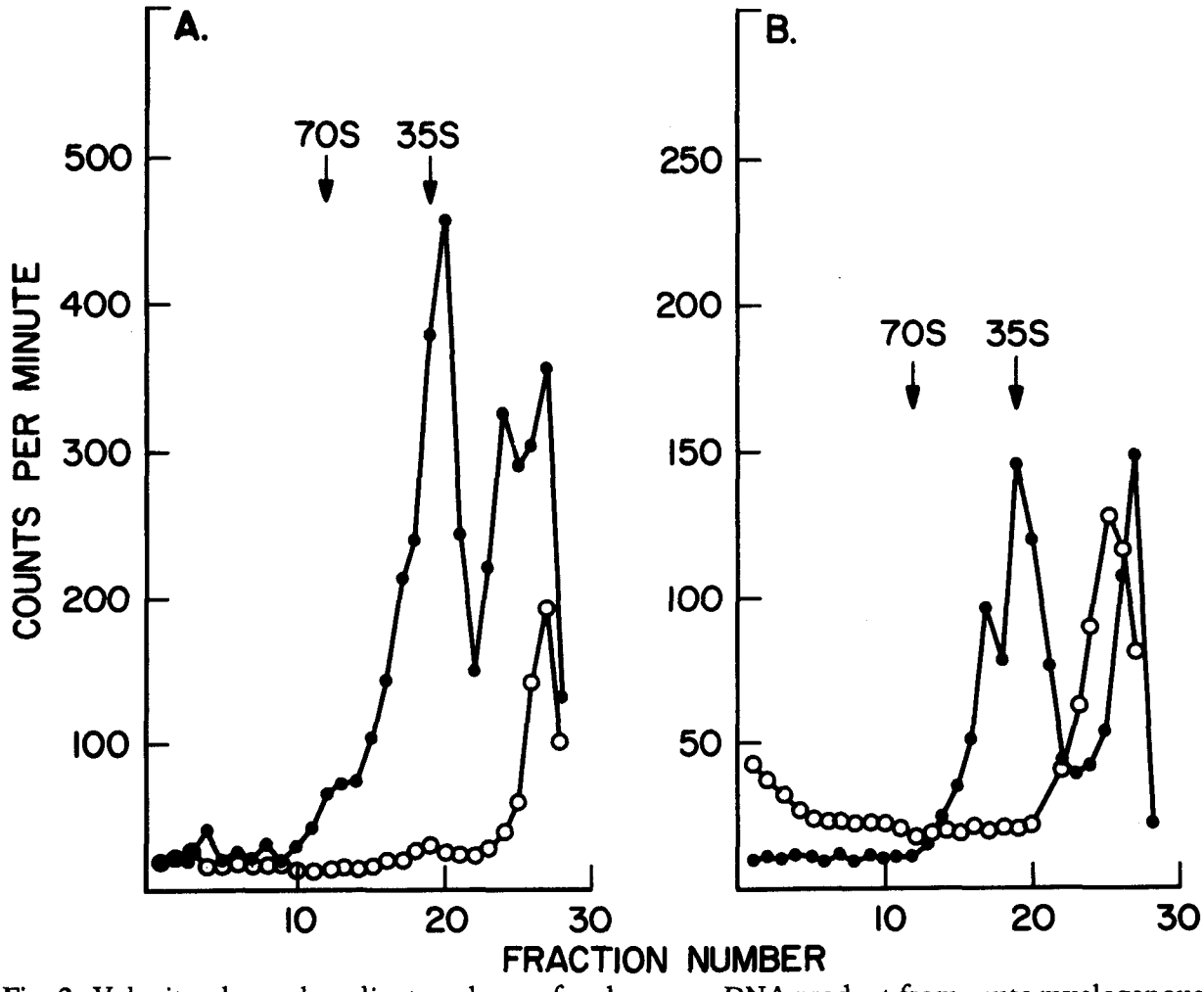

Fig. 2: Velocity glycerol gradient analyses of endogenous DNA product from acute myelogenous leukemia cells (G. F.). Procedures are described in Fig. 1. (A) Endogenous DNA product from the 98,000 xg "crude" cytoplasmic pellet fraction: ●——●, all 4 trinucleotides present in the reaction mixture; ○——○, dGTP omitted from the reaction mixture. (B) Endogenous DNA product synthesized by the 1.15 to 1.17 density fraction after sucrose equilibrium density gradient centrifugation (21): ●——●, untreated; ○——○ after RNase treatment.

Table 2. Velocity gradient analyses of ^{3}H-DNA products from leukemic blood cells after short-term culture*

Patient	Diagnosis	Rapidly Sedimenting (RS) DNA			Results From Fresh Cells
		Estimated Size	cpm RS DNA	Per Cent Total cpm	
N.N.	AML	35, 70S	98, 83	12.4, 10.6	N.D
G.F.	AML	70S	180	10.5	35S
J.C.	AMML	35S	795	15.3	N.D.
C.P.	AMML	35S	135	2.6	35S
J.Wh.	CML	35S	101	5.2	N.D.

* Leukemic blood cells were placed in stationary culture at 1 x 10^7 cells/ml, using RPMI 1640 containing 20 % fetal calf serum, at 37 °C for 6 to 8 days. All methods and designations are given in Table 1.

RNA from purified RNA tumor viruses. Occasionally, as in Figure 1, some RNase-sensitive ^{3}H-DNA complex was noted at the bottom of the glycerol gradient and in the 12 to 28S region of the gradient. The former probably represents molecular aggregates; the latter may represent DNA associated with partially degraded RNA template, although other explanations are possible.

In several instances, 0.5 to 1.0 gm of leukemic cells were placed in short-term tissue culture (6 to 8 days) before preparing endogenous DNA product for glycerol gradient analysis. After culture, DNA product from three leukemic cytoplasmic pellet preparations which had previously failed to show any rapidly-sedimenting DNA, was demonstrated to sediment in part as ribonuclease-sensitive 35S and 70S complexes (Table 2). Similar results were obtained in the presence of 5′ – iodo-deoxy-uridine (20 μg/ml for the first 48 hours in culture), a compound capable of "inducing" leukemia virus from non-producing mouse tissue culture cells (10).

Overall, in 7 of 8 cases of leukemia involving myeloblastic cells endogenous DNA product was detected as a complex with RNA characteristic in size of intact (70S) or

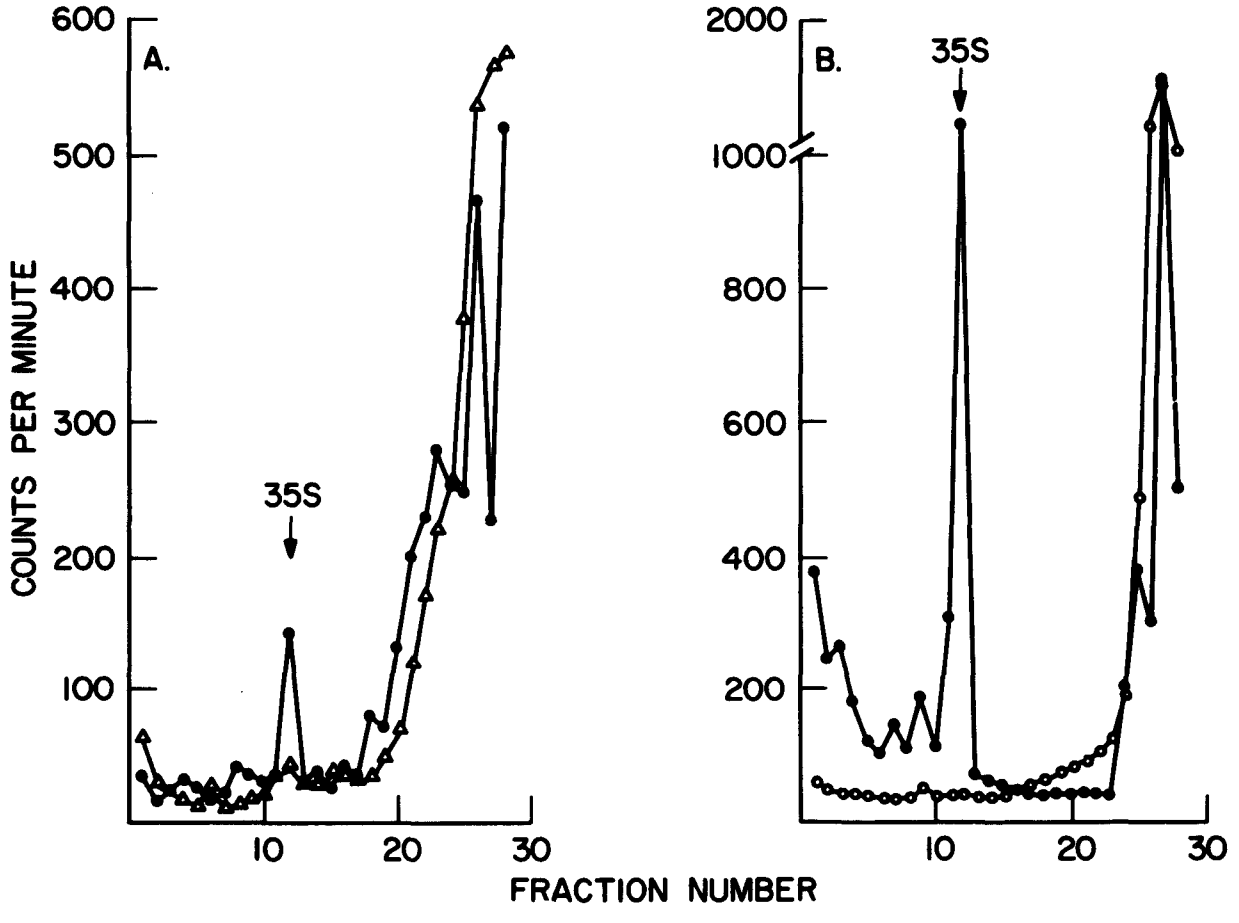

Fig. 3: Velocity glycerol gradient analyses of endogenous DNA product from acute myelomonocytic leukemia cells (C. P.). Procedures are described in Fig. 1. (A) Endogenous DNA product from the 98,000 xg "crude" cytoplasmic pellet fraction: ●——●, untreated; △——△, after ribonuclease treatment. (B) Endogenous DNA product synthesized by the 1.13 to 1.15 density fractions after equilibrium sucrose gradient centrifugation (21): ●——●, all 4 trinucleotides present; O——O, dGTP omitted from the reaction mixture.

subunit (35S) RNA from RNA tumor viruses. Similar analyses of three sets of normal, peripheral blood buffy coat leukocytes and three sets of phytohemagglutinin-stimulated lymphocytes showed no evidence of rapidly sedimenting DNA-RNA complexes. These data confirm the report by Baxt, et al. (5), that nearly all human acute

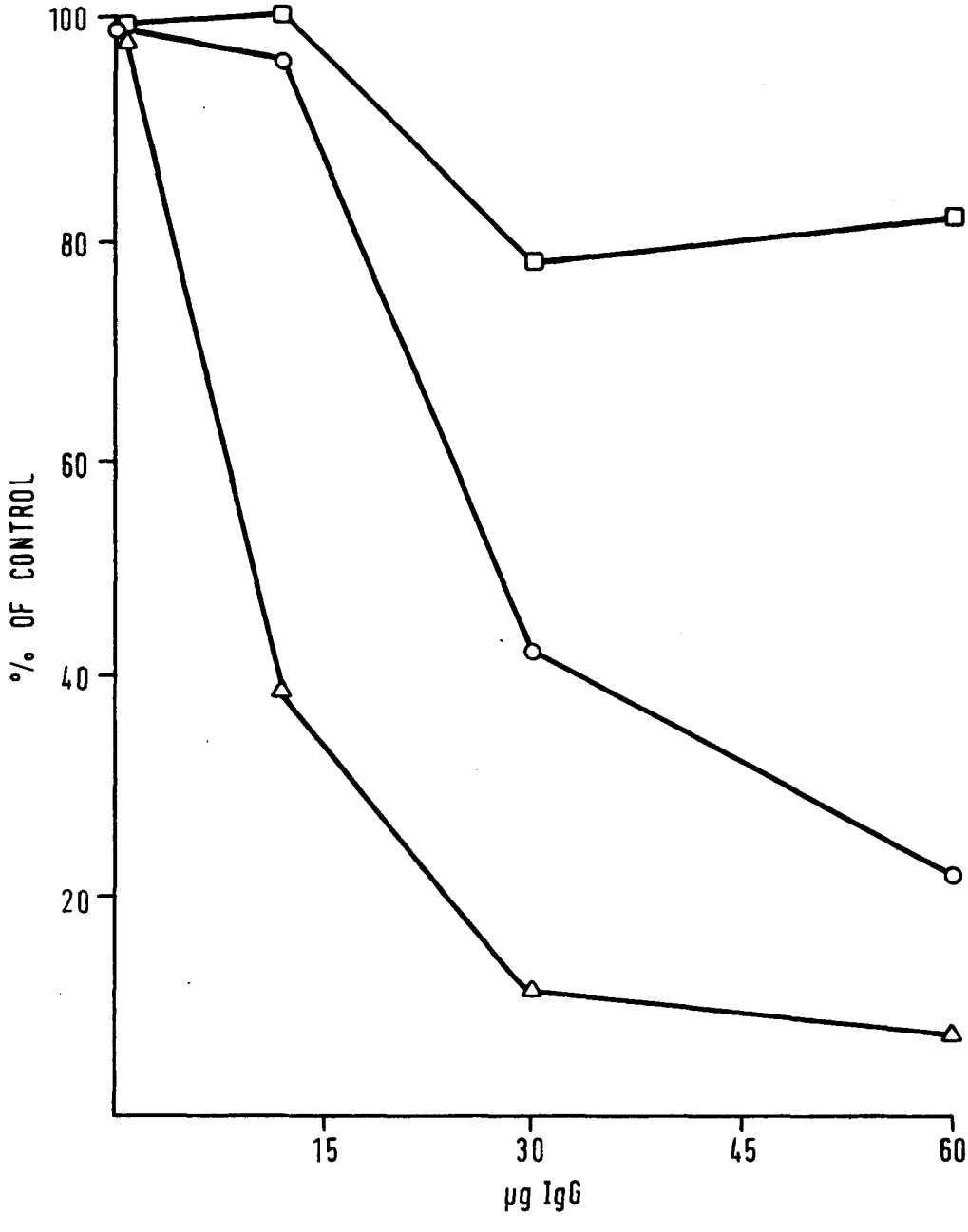

Fig. 4: The effect of variable concentrations of IgG from antisera to reverse transcriptase from primate and mouse C-type viruses on the leukemic cytoplasmic pellet enzyme. The preparation of the post-sepharose enzyme used in this experiment is outlined in the text, and detailed procedures for the preparation of antisera and for the performance of enzyme reactions are presented elsewhere (3, 21). □——□, murine leukemia virus (Rauscher) antipolymerase IgG; ○——○, gibbon ape lymphosarcoma virus antipolymerase IgG; △——△, simian sarcoma virus antipolymerase IgG.

myelogenous leukemia cells contain RNA which is the same size as RNA from RNA tumor viruses, and the template function of this RNA with its associated DNA polymerase, verifies (by a different approach) the earlier reports from our laboratory of reverse transcriptase in human leukemic cells (1–3).

Molecular Hybridization of Endogenous DNA Product

Endogenous DNA product, synthesized from the cytoplasmic pellet fraction of patient G. F. (AML) in the presence of actinomycin D (50 μg/ml), was shown to be complexed approximately 50 % to 35S RNA by velocity glycerol gradient analysis (Figure 2A; Table 1). After further purification of this pellet fraction by equilibrium sucrose density gradient centrifugation, the particulate fraction synthesizing DNA product complexed with 35S RNA was localized at a density of 1.15 to 1.17 g/ml (Figure 2B), the density characteristic of RNA tumor viruses. DNA product synthesized from this sucrose density fraction was annealed for 8 days to denatured viral RNA immobilized on 7 mm phoshocellulose discs (11). As shown in Table 3, over half of the input DNA hybridized to 70S RNA from simian sarcoma virus (SiSV; derived from a woolly monkey fibrosarcoma; 12). Approximately, 20 % and 5 % of the input DNA was bound, respectively, to murine sarcoma virus (Kirsten) and murine leukemia virus (AKR) RNA. Further information related to DNA product from patient G. F. and hybridization data with similar results from a patient with chronic myelogenous leukemia in blast crisis (J. Wh.) are presented in detail elsewhere (4). The pattern and magnitude of these hybridization results leave little doubt that RNA from the cytoplasmic pellet fraction of human myelogenous leukemia cells have nucleotide sequences in common with genomic RNA from RNA tumor viruses.

Identification of an RNA Tumor Virus-Related RNA-Directed DNA Polymerase in the Endogenous, DNA Synthesizing Cytoplasmic Pellet Fraction

Biochemical Properties

Endogenous DNA product from the cytoplasmic pellet fraction of patient C. P. (AMML), was complexed to 35S RNA, and the proportion of 35S-sedimenting DNA synthesized was considerably enriched after sucrose density-gradient banding of the pellet fraction (Figure 3). This enrichment phenomenon has been observed in several cases and is probably due to purification of the RNA and reverse transcriptase containing particulate fraction from contaminating degradative enzymes. Similarly, the endogenous, ribonuclease-sensitive DNA polymerase activity from the pellet fraction has also been augmented by sucrose gradient banding, and in several cases, marked purification of this activity at a density of 1.14 to 1.18 has occured after repetitive sucrose gradient bandings (4), suggesting the presense of discrete virus-like particles in human leukemic cells.

In the present case (C. P.), DNA polymerase activity which slightly preferred the synthetic template-primer $(A)_n \cdot dT_{12-18}$ to $(dA)_n \cdot dT_{12-18}$ (1.45 to 1) was observed in the 1.13 to 1.15 g/ml sucrose density fractions. The DNA polymerase activity favoring RNA template $(A)_n$ was further purified, not by convential techniques for

the purification of polymerase enzymes, but by sepharose 4B chromatography, a procedure for analyzing high molecular weight molecules or complexes (exclusion = 20 x 10^6 daltons). After this procedure, the endogenous, ribonuclease-sensitive DNA polymerase activity and most of the DNA polymerase activity preferring $(A)_n \cdot dT_{12-18}$ (now by 8 : 1 over $(dA)_n \cdot dT_{12-18}$) was localized in the exclusion volume of the sepharose column, indicating the presence of a large complex containing 35S RNA and RNA-directed DNA polymerase.

When reaction conditions were optimized by adjusting the MnC1 and KC1 concentrations, respectively, to 1 mM and 100 mM, the preference of the post-sepharose enzyme for $(A)_n \cdot dT_{12-18}$ over $(dA)_n \cdot dT_{12-18}$ increased to greater than 40 : 1 (Table 3). This enzyme, subsequently termed the "leukemic pellet enzyme," had an absolute requirement for divalent cation with Mn++ markedly preferred to Mg++. $(dA)_n \cdot dT_{12-18}$ was poorly utilized under all reaction conditions tested. This clearly differentiates the leukemia pellet enzyme from DNA polymerases I and II from normal leukocytes, which strongly prefer $(dA)_n \cdot dT_{12-18}$ over $(A)_n \cdot dT_{12-18}$ (13). This criterion does not differentiate between the leukemic enzyme and the "R-DNA polymerase," which has been demonstrated in several types of mammalian cells by Weissbach and his associates (14). However, as further shown in Table 3, the leukemic pellet enzyme utilized $(C)_n \cdot dG_{12-18}$ approximately 30 %, and in repeat studies up

Table 3. Hybridization of the (^{3}H)-DNA Product of the Endogenous RNA-Directed DNA Polymerase from Human Leukemic (GF) Cytoplasmic Pellet Fraction to RNA Isolated from RNA Tumor Viruses*

RNA+	cpm‡	cpm (−AMV)	% Hybridization
SiSV (NRK)	550	450	53
MuSV (Kristen)	259	159	19
MuLV (AKR)	145	45	5
AvLV (AMV)	100	–	–
Input	840	–	–

* ^{3}H-DNA product was synthesized from the cytoplasmic pellet fraction, banding at a density of 1.16 g/ml, after equilibrium sucrose density gradient centrifugation (4). This DNA product was purified and annealed for eight days in 0.1 ml of 50 % formamide- 3XSSC (final concentrations) to 0.2 μg of immobilized 70S viral RNA (4, 11).

+ The tumor viruses used as a source of RNA are abbreviated as follows: SiSV (NRK) = Simian sarcoma virus grown in normal rat kidney cells (RK); (Kirsten) = a sarcoma-leukemia virus complex grown in NRK cells which originated by repeated infection of rats with a Gross-type mouse leukemia virus; MuLV (AKR) = a Gross-type mouse leukemia virus grown in mouse fibroblast cells and originating spontaneously from AKR mice; AvLS (AMV) = avian leukosis virus, strain avian myeloblastosis.

‡ Data are presented as crude filter-bound radioactivity in counts per minute (cpm), as the same data corrected for radioactivity trapped by a heterologous RNA (cpm minus AMV), or as the corrected data expressed as percent of the input DNA recovered in a hybrid structure (% hybridization).

Table 4. Template-Primer Specificity of the Leukemic Cytoplasmic RNA-Directed DNA Polymerase After Sepharose 4B Purification*

Additions	^{3}H-Deoxyribonucleotide Triphosphate	pmole Incorporated+ X 10^{-1}
$(A)_n.dT_{12-18}$	TTP	7.56
$(dA)_n.dT_{12-18}$	TTP	0.17
$(C)_n.dG_{12-18}$	dGTP	2.13
dT_{12-18}	TTP	0.35
	dGTP	0.01
dG_{12-18}	TTP	0.08
	dGTP	0.06

* Methods for the preparation of the post-sepharose enzyme are indicated in the text and described in detail in Ref. 21.

+ The pmoles of TMP or dGMP incorporated in 30 minutes at 37 °C in a 0.05 ml reaction mixture containing 50 mM Tris, pH 8.3, 1 mM $MnCl_2$, 3 mM dithiothreitol, 6 μM ^{3}H-TTP (54 Ci/mMole) or ^{3}H-dGTP (10 Ci/mMole), 0.01 ml of the post-sepharose enzyme ("activated" by pre-incubation for 10 minutes at 37 °C in 0.1 % Triton X-100), and 3860 pMoles (expressed as pmoles of mononucleotide) of oligomer (dG_{12-18} or dT_{12-18}) with or without an equimolar amount of complementary polymer ($[C]_n$, $[A]_n$, or $[dA]_n$).

to 50 %, as well as $(A)_n \cdot dT_{12-18}$. This level of activity with $(C)_n \cdot dG_{12-18}$ has previously been observed only with true viral reverse transcriptase (3, 14, 15). Recently, Fry and Weissbach reported the use of $(C)_n \cdot dG_{12-18}$, approximately 5 % as well as $(A)_n \cdot dT_{12-18}$, by R-DNA polymerase from mouse L-cells (15); this result must be interpreted with reservation since mouse L-cells are infected with RNA "tumor" virus (16). We find that extensive utilization of this template-primer is a specific indication of virus polymerase, in agreement with Baltimore and his colleagues (3, 17, 18). Finally, since no significant activity was demonstrated with the primers dT_{12-18} and dG_{12-18} (Table 3), this enzyme is not terminal deoxynucleotidyl transferase, which was recently reported to be present in leukemic cells from one patient with acute lymphocytic leukemia (19). Moreover, as shown in Table 4, there is no activity with primer alone (i. e., without templates), the assay used for detection of terminal transferase (19).

Immunological Properties

In order to further investigate the relatedness of the leukemic reverse transcriptase to viral reverse transcriptase, we tested for possible inhibition of the enzyme by purified IgG from antisera prepared against reverse transcriptases from various RNA tumor viruses. As shown in Figure 4, the leukemic enzyme was markedly inhibited by antipolymerase IgG to the two known primate C-type viruses, simian sarcoma virus (12) and gibbon ape lymphosarcoma virus (20). A slight degree of inhibition was noted with antipolymerase IgG against reverse transcriptase from mouse leukemia

virus (Rauscher) (Figure 4). In other studies, antisera to reverse transcriptase from avian sarcoma virus and the non-C-type Mason-Pfizer monkey virus did not inhibit the leukemic enzyme. These results, which are reported in greater detail elsewhere (21), are very similar in pattern to those recently reported for RNA-directed DNA polymerase from another case of acute myelogenous leukemia (3). We have further demonstrated that these leukemic enzymes are not inhibited by antisera to DNA polymerase I from phytohemagglutinin-stimulated lymphocytes (23).

These immunological results confirm the biochemical data by demonstrating that the human leukemic reverse transcriptase is related to virus reverse transcriptase but not to normal cellular DNA polymerase I. It seems probable that the leukemic enzyme is, also, not related to cellular DNA polymerase II (13), since antisera to reverse transcriptase from RNA tumor viruses do not inhibit either of the major cellular DNA polymerases (22). The immunologic results extend the biochemical data by demonstrating that the leukemic enzyme has close homology to reverse transcriptase of C-type viruses from primates but not from lower species. These results are similar to those reported from a study of the immunologic relationships of primate oncornaviruses and agree with the specificity of the nucleic acid hybridization results previously mentioned (4).

Discussion

The data presented here indicate with near certainty that human acute myelogenous leukemic cells contain RNA and RNA-directed DNA polymerase related in function and structure to genomic RNA and to reverse transcriptase from RNA tumor viruses, particularly from primate C-type viruses. These findings make "evolutionary sense" in that both the RNA and RNA-directed DNA polymerase are related in general biochemical function to analogous molecules in distantly-related species but by exact, primary structural criteria (nucleic acid hybridization and immunologic cross-reactivity) have extensive homology specifically to RNA and reverse transcriptase from closely-related, primate species. Although these data do not establish the cause of human myelogenous leukemia, they do provide powerful support for the concept that C-type viruses have an etiologic role in leukemia in man, as in other species. Also, these data do not indicate whether the leukemic viral information comes from endogenous genetic information or from exogenous sources. The finding of similar nucleic acid hybridization patterns of leukemic DNA product (4) and of similar immunologic reactivity patterns of "reverse transcriptase" from different patients with myelogenous leukemia suggests that a common viral element may be involved, although more discriminating studies with specific molecular probes are required to resolve this issue.

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