INHIBITION OF PROTEIN SYNTHESIS IN RETICULOCYTE LYSATES BY DOUBLE-STRANDED RIBONUCLEIC ACID EXTRACTED FROM NUCLEI OF LEUKEMIC CELLS

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1. Introduction

It has been recently shown in our laboratory [1] that human leukemic blast cells synthesize in their nuclei, not unlike several other types of animal cells [2–6], significant amounts of RNA with double-stranded properties. The functional role of these sequences is at present unknown. However, one of the most interesting properties of double-stranded RNA (dsRNA) is the ability of very small amounts to inhibit protein synthesis in cell-free systems. So far, this property has been shown mainly for viral dsRNA [7–11], although HeLa cell dsRNA also appears to inhibit "in vitro" protein synthesis [12]. In the present paper we report the results of experiments carried out in our laboratory indicating that dsRNA extracted from nuclei of human leukemic blast cells efficiently inhibits protein synthesis in reticulocyte lysates.

2. Material and methods

Leukemic blast cells were obtained from the circulating blood of three untreated patients with acute leukemia (two of them had acute lymphatic leukemia and one had acute myeloid leukemia) having high levels of circulating leukocytes. Red cells were sedimented by gravity and the supernatant was diluted with autologous plasma and Eagle's Minimal Essential Medium to obtain a final concentration of 3×10^6 cells per ml with 20% plasma. [^{3}H]Uridine (Radiochemical Centre, Amersham) was then added at a final concentration of 20 μCi/ml and the cells were havested after 3 hr of incubation at 37°C.

2.1. *Nuclei isolation*

Nuclei of high purity were obtained without mechanical shearing by a minor modification of the method of Takakusu et al. [13], using a mixture of detergents solutions containing sodium deoxycholate and Triton WR 1339. The harvested cells were washed twice with 50 ml of sucrose buffer (0.15 M sucrose, 0.005 M $CaCl_2$, 0.05 M Tris–HCl, pH 7.1). The washed cells were resuspended in 15 ml of the same buffer, and an equal volume of detergent solution (0.25% sodium deoxycholate, 0.5% Triton) was added, and the suspension shaken gently by hand for 5 min. The suspension was then diluted with 100 ml of sucrose buffer to prevent further detergent activity, and then centrifuged at 1500 rev/min for 10 min, to eliminate the cytoplasmic components in the supernatant. The resulting pellet of nuclei was washed thrice with 20 ml of 0.25 M sucrose buffer at 4°C. Microscopic examination of Giemsa stained nuclei showed that the nuclear chromatin and nucleolar structure were well preserved, and that only a very few nuclei retained small cytoplasmic tags.

2.2. *RNA extraction and dsRNA separation*

RNA was extracted from a 0.5 ml nuclear pellet by hot phenol-m-cresol, as already described [14], and precipitated with 75% ethanol. The resulting pellet, washed twice with 5 ml of 3 M Na-acetate, was dissolved in 5 ml of buffer (50 mM NaCl, 2 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.4) and digested for 30 min at room temperature with DNase (20 μg/ml). 30 μg/ml of pancreatic RNase and 20 units/ml of T1 RNase were then added and the solution incubated for 30 min at 37°C. Following incomplete digestion, the nucleic acid was extracted once more with phenol-

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m-cresol and precipitated again with ethanol. To separate dsRNA, CF 11 cellulose columns were used, according to the method of Franklin [15]. The precipitated nucleic acid was dissolved in 0.5 ml of buffer (0.1 M NaCl, 1 mM NaEDTA, 50 mM Tris–HCl, pH 6.9) containing 35% ethanol. The sample was applied to a cellulose column (10 cm × 1 cm) and the column was washed with 75 ml of the same solution, followed by 75 ml of buffer containing 15% ethanol. DNA, transfer RNA and single-stranded RNA were so eluted. dsRNA was finally recovered by washing the column with buffer alone. 5 ml fractions were collected, and radioactivity was determined on 0.5 ml aliquots of each fraction. Three most radioactive fractions of third peak from each one of four nuclear pellets were pooled, and RNA was precipitated by adding 2.5 vol of ethanol and 100 μg of *E. Coli* transfer RNA as carrier. After centrifugation, the precipitate was dissolved in 0.5 ml of buffer and the solution was tested in the synthetic reaction.

2.3. *Preparation of lysates*

Reticulocytes were obtained from rabbits of about 3 kg made anemic by injecting subcutaneously with 2 ml of 1.25% of acetylphenylhydrazine on four successive days, according to Hunt and Jackson [16]. Five days later they were bled by cardiac puncture, using heparin as anticoagulant, and the reticulocytes constituted about 50% of the red cells present in the blood. These cells were washed three times with ice cold saline (0.13 M NaCl, 5 mM KCl, 7.5 mM $MgCl_2$) and lysed with 1.5 vol of ice-cold water per vol of packed cells. The debris were removed by centrifugation at 14 000 rev/min in the SW 25.1 rotor of a Beckman ultracentrifuge. 1 ml aliquots were stored at −100°C.

2.4. *Incubation for protein synthesis*

In order to obtain protein synthesis the lysate was thawed and hemin was added at a final concentration of 20 μM, according to the procedure of Hunt and Jackson [16]. The incubation mixture was then prepared, containing the following components: 0.8 ml of lysate, 0.05 ml of salt solution, (2 M KCl, 10 mM $MgCl_2$), 0.05 ml of 0.2 M creatine phosphate, and 0.05 ml of an amino acid mixture with the following composition: Arg 0.5 mM; Asn 0.5 mM; Asp 2 mM; Cys 0.5 mM; Gln 0.5 mM; Glu 2 mM; Gly 2 mM; His 2 mM; Ile 0.5 mM; Leu 3 mM; Lys 2 mM; Met 0.5 mM; Phe 1.5 mM; Pro 1 mM; Ser 2 mM; Thr

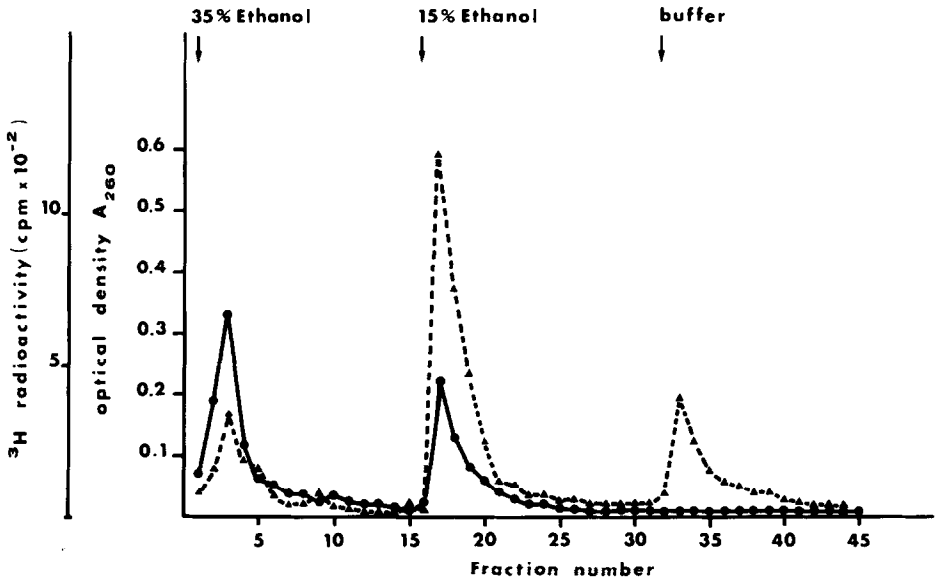


Fig.1. Optical density and radioactivity elution profiles obtained by chromatography on CF 11 cellulose column of a sample of leukemic cell nuclear RNA after digestion with DNase and RNase. (●——●) Optical density; (▲-----▲) [^{3}H]radioactivity in each 5 ml column fraction.

1.5 mM; Try 0.5 mM; Tyr 0.5 mM; Val 3 mM. Alanine was omitted since [^{14}C]alanine was used as a label. Before addition to the lysate the amino acid mixture was neutralized with KOH to pH 7.5 and creatine kinase was added at a final concentration of 0.1 mg/ml. 50 μ aliquots of either RNA solution or buffer alone were added to 400 μl of the assay mixture and incubated for 10 min at 33°C before addition of 50 μl of [^{14}C] alanine solution at the final concentration of 50 μCi/ml. 20 μl aliquots were then removed at 4 min interval into 2 ml of distilled water, and 0.2 ml of H_2O_2 36% were added in order to remove the color of hemoglobin. 1 ml of 1 N NaOH containing 1 mg/ml of unlabeled alanine was added, the samples were incubated for 15 minutes at 37°C and protein was precipitated with TCA 8% before filtration on Whatman GF/C glass fiber filters. ^{14}C counting was performed with a Packard TriCarb liquid scintillation spectrometer.

3. Results

Although very little optical density was eluted with buffer alone, as shown in fig.1, a significant amount of radioactivity was still present in this eluate. As indicated originally by Franklin [15], and afterwards confirmed by several authors [3,6,11], this [^{3}H]uridine-labeled material is mainly dsRNA, which remains attached to the cellulose during stepwise washes with buffer containing 35% and 15% ethanol. The presence of labeled RNA in the third chromatographic peak was confirmed by the following results: (1) all the radioactivity became acid soluble after hydrolysis with 0.3 M NaOH; not more than 10% of the third peak radioactivity was made acid soluble by pancreatic RNase: however, when a sample was heated 5 min at 100°C, chilled quickly in an ice-water bath, brought to 0.25 M total NaCl and digested 30 min at 37°C with RNase (20 μg/ml), all the radioactivity became acid-soluble.

The ability of dsRNA eluted in buffer without ethanol to inhibit protein synthesis is shown in fig.2, indicating the kinetics of protein synthesis in the presence of two samples of dsRNA obtained from cells of two different patients. After an initial phase, in which the initial rate of incorporation of the labeled amino acid remained unaffected, a second phase followed, characterized by the abrupt cessation of protein synthesis. This behavior is now considered typical of the effect of dsRNA on protein synthesis in vitro [10]. Control experiments showed that transfer RNA, which had been used as carrier, had

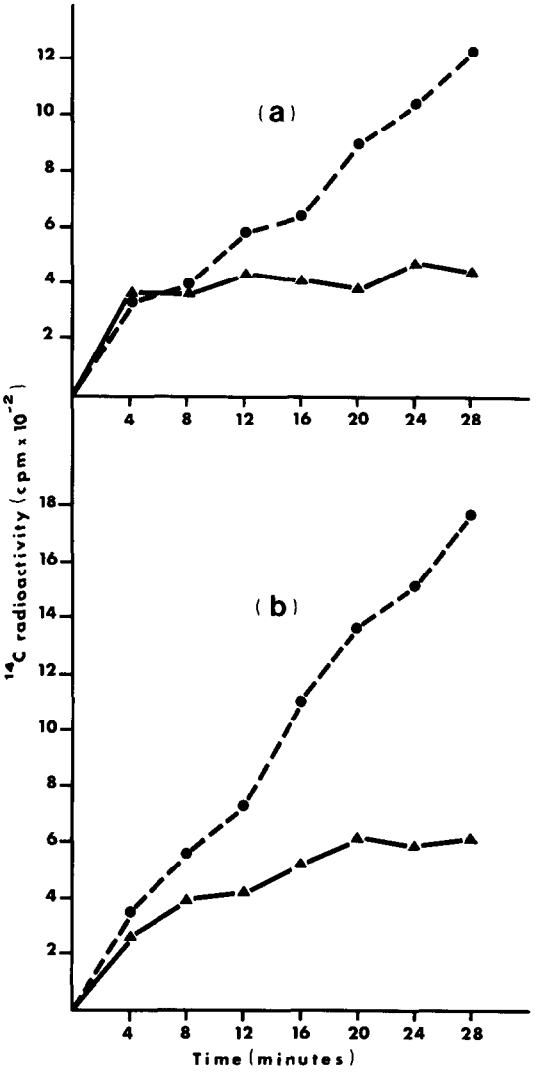
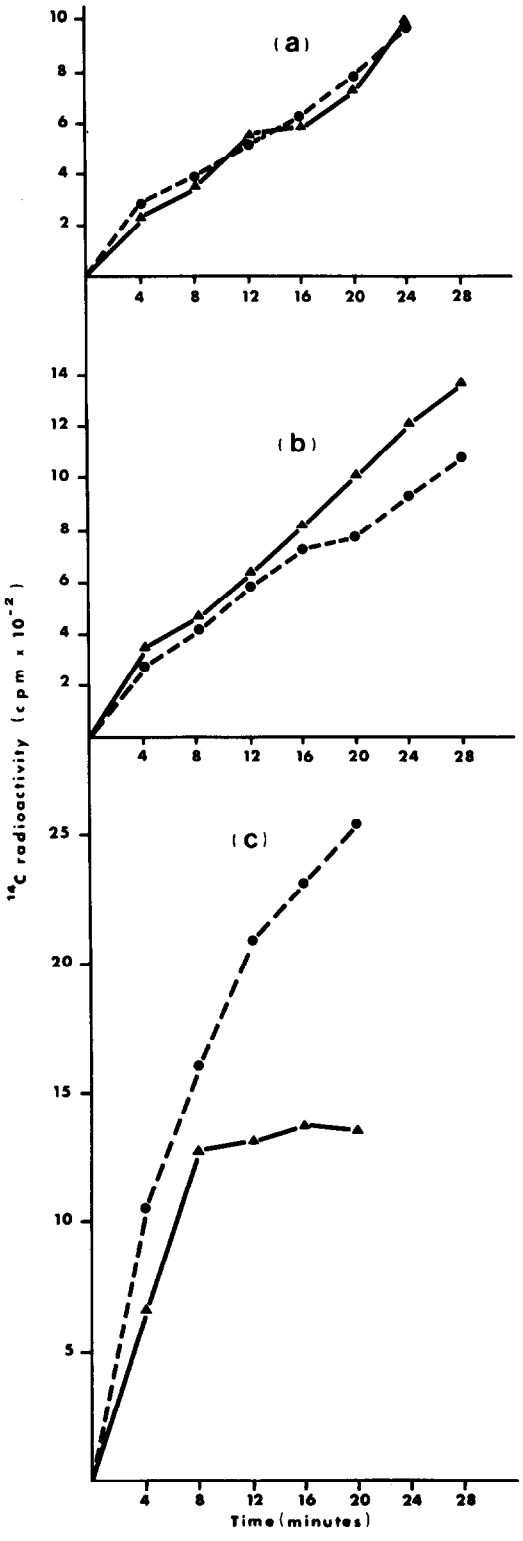


Fig.2. Kinetics of protein synthesis in the reticulocyte lysate system in the presence of two different samples of dsRNA extracted from nuclei of blast cells of a case of acute myeloid leukemia(a) and acute lymphatic leukemia(b). In vitro protein synthesis was carried out as described in Materials and methods. 20 μl aliquots were removed at the indicated times and precipitated and counted as described. (▲——▲) Incubation in the presence of 50 μl of 50 μl of pooled dsRNA from several eluates as described in the text; (●- - -●) Incubation in the presence of 50 μl of buffer.



no effect on protein synthesis (fig.3a) even at the concentration of 5 μg/ml. Some polyadenylate sequences appear to cochromatograph with dsRNA on cellulose CF 11 columns [6,11], although most of these sequences elute with buffer containing 15% ethanol. We have thus assayed the effect of synthetic poly(A) on our in vitro system, showing that no inhibitory action may be observed at the concentration of 5 μg/ml (fig.3b). On the contrary, strong inhibition was observed with synthetic poly(I): poly(C) at the concentration of 0.025 μg/ml (fig.3c).

4. Discussion

The presence in the third eluate of [^{3}H]uridine-labeled material resistant to RNase already strongly suggested the existence, in leukemic cell nuclear RNA. of regions involved in complementary base paring. The inhibitory action of this material on protein synthesis by reticulocyte lysate represents further evidence that this RNA includes double-stranded segments. In fact, inhibition of globin synthesis has been recently indicated as the most sensitive method to distinguish between double-stranded and single-stranded RNA, since it responds to concentration of dsRNA as low as 0.1 μg/ml [11]. The biphasic kinetics of protein synthesis and the very low concentration of our inhibitory material make very unlikely that inhibition in our system is caused by polynucleotides other than dsRNA. No indication is however obtained about the size of these RNA segments, since the inhibitory activity does not vary with a size above the minimum length of at least 50 base pairs [10].

Fig.3. Kinetics of protein synthesis in the reticulocyte lysate system in the presence of different types of RNA. (a) Incubation in the presence of *E. coli* tRNA 5 μg/ml. (b) Incubation in the presence of synthetic poly(A), 5 μg/ml. (c) Incubation in the presence of Poly (I): poly (C), 0.025 μg/ml. (▲——▲) Incubation in the presence of 50 μl of RNA solution; (●- - -●) Incubation in the presence of 50 μl of buffer.

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