APPEARANCE OF REVERSE TRANSCRIPTASE ACTIVITY IN HUMAN FETAL THYMUS CELLS STIMULATED BY HUMAN B-CELL^{*1}

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ABSTRACT

We have investigated three human fetal thymuses by various induction procedures in a search for retrovirus. Thymus cells were cultured for 7 days together with mitomycin C-treated B cells and examined for reverse transcriptase activity. The following results were obtained. 1) RNA-directed DNA polymerase (RDDP) activity was detected in the culture medium on day 3 and persisted until day 7. This enzyme, after partial purification on phosphocellulose column, showed template-primer specificity of a typical reverse transcriptase. 2) In unstimulated thymus cells, no reverse transcriptase activity was detected. 3) Stimulation with lymphocyte mitogens such as phytohaemagglutinin (PHA) and concanavalin A (Con A) exerted no effect on inducing the reverse transcriptase activity. 4) No reverse transcriptase activity was obtained in the culture medium of B cells pretreated with mitomycin C. Taken together these results indicate that in human fetal thymus cells reverse transcriptase activity is induced by human B cell stimulation, pointing to an intriguing possibility of protovirus activation.

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RNA tumor viruses (retroviruses) have been shown to be involved in the pathogenesis of leukemias, lymphomas, sarcomas and so forth in several species of animals.¹⁻³ Most of retroviruses are considered to be endogenous, since they are induced in normal cells and since molecular hybridization studies have shown that their proviruses are integrated into the cell genome.⁴⁻⁶ In humans, several authors have detected retroviruses in cells of malignant tumors such as leukemias⁷⁻¹⁰ and also in normal tissues such as placentas.^{11,12} However, an inducible, endogenous retrovirus has not yet been demonstrated in humans.

In order to determine whether the findings in animals could be applied to humans, we searched for endogenous retrovirus in human thymus cells by various induction procedures. The reasons why thymus cells were chosen were: 1) The thymus is considered to be the primary site of neoplastic transformation in spontaneous leukemia in AKR mice.¹³ 2) The existence of Tla (thymus-leukemia antigen) on murine thymus cells.¹⁴ 3) Retroviruses have been detected in human adult T-cell leukemias^{15,16} and the thymus is considered to be a central lymphoid tissue.¹⁷

In this paper, we present evidence for the appearance of reverse transcriptase activity in human fetal thymus cells. As judged by template-primer specificity and chromatographic behavior on phosphocellulose column, the enzyme was shown to be an RNA-directed DNA polymerase (RDDP) and such reverse transcriptase activity is seen in human fetal thymus cells only when they are stimulated by human B cells. The results suggest the intriguing possibility that RNA tumor provirus can be activated in normal human thymus cells.

MATERIALS AND METHODS

Cells and Cell Culture Methods

Three human fetal thymuses were obtained by abortion (from 14 to 20 weeks old) and thymus cell suspensions were prepared as described.^{18,19} These suspensions consisted of 70-90% thymic lymphocytes and 10-30% non-lymphoid cells including thymus epithelial cells. Two B cell lines, CCRF-SB,²⁰ Raji,²¹ were used as stimulator cells. The thymus cells (5×10^6 cells/well) were cultured for 7 days at 37°C in an atmosphere of 5% CO₂/95% air together with B cells (1×10^6 cells/well) pretreated with mitomycin C (Kyowa, 25 µg/ml, 37°C, 30 min). On day 4, a second stimulation by B cells was applied. On day 1, 3, 5 and 7 the culture medium was collected and processed for the assay of RNA-directed DNA polymerase (RDDP) activity. The following induction procedures were also applied to fetal thymus cells, culturing with either phytohaemagglutinin-P (1 µg/ml), concanavalin A (10 µg/ml), lipopolysaccharide (10 µg/ml) or pokeweed mitogen (10 µg/ml), and pretreatment with bromodeoxyuridine (BUDR,

50 $\mu\text{g/ml}$, Difco) or mitomycin C. The culture medium was collected on day 3 and examined for RDDP activity. In order to exclude the possibility that the stimulator B cells were contaminated with retroviruses, both fresh and mitomycin C treated B cells were checked for RDDP activity. B cells pretreated with BUDR (100 $\mu\text{g/ml}$) were also studied in the same way. As a control of RDDP activity, murine leukemia virus (MuLV) producer cell lines were used.²²

Partial Purification of the RDDP Activity

The culture medium was collected and centrifuged at $2,000\times g$ to remove cell debris. The supernatant was centrifuged at $105,000\times g$ for 90 min at 4°C and the pellet was resuspended in buffer A (0.05 M Tris-HCl, pH 7.5, 50 mM KCl, 5 mM MgCl_2 , 5 mM dithiothreitol). An aliquot of this suspension was placed onto the top of 20–60% w/v sucrose gradients in buffer A and centrifuged for 18 hrs at $150,000\times g$ at 4°C .²³ Fractions (0.25 ml) were collected from the bottom and assayed for RDDP activity. The density of each fraction was determined by measuring the refractive index. Sucrose density gradient fractions containing RDDP activity were pooled and mixed with an equal volume of disruption buffer (0.05 M Tris-HCl, pH 7.5, 1.0 M KCl, 1.0% Triton X, 5 mM dithiothreitol, 20% glycerol). The pooled fractions were briefly sonicated, stirred at 4°C for 3 hrs and centrifuged at $100,000\times g$ for 1 hr. The supernatant fluid was dialyzed against buffer B (0.05 M Tris-HCl, pH 7.5, 5 mM dithiothreitol, 20% glycerol) containing 0.05 M KCl. The dialyzate was then chromatographed on a DEAE column (8×1.4 cm, Whatman, DE 52) equilibrated with buffer B containing 0.05 M KCl.²⁴ The column was developed with a 60 ml linear 0.05 M–0.5 M KCl gradient in buffer B. Fraction (3 ml) were collected and assayed for DNA polymerase activity. Fractions containing enzyme activity were pooled and dialyzed against buffer B containing 0.05 M KCl. The dialyzed solution was then applied to a phosphocellulose column (8×1.4 cm, Whatman P 11) equilibrated with buffer B containing 0.05 M KCl. The column was developed with a 60 ml of linear 0.05–0.6 M KCl gradient in buffer B. Fractions (3 ml) were collected and assayed for DNA polymerase activity. As a control, post-mitochondrial supernatant prepared from fresh thymus or B cells or MuLV producer cells was sonicated and stirred in the same manner. After dialysis the solution was chromatographed on a phosphocellulose column, and assayed for DNA polymerase activity.

Assays for RDDP and other DNA Polymerase Activities^{23,25}

RDDP assays were carried out at 37°C for 30 min in a standard reaction mixture which contained in a final volume of 100 μl : 50 mM Tris-HCl (pH 7.8), 60 mM KCl, 1 mM MnCl_2 , 5 mM dithiothreitol, 0.1% (v/v) Triton X-100, 4 μM

^3H IdTTP (30 Ci/mM, 60,000 cpm/pmole), $2\text{ }\mu\text{g}$ $(\text{rA})_n \cdot (\text{dT})_{12-18}$ and a $50\text{ }\mu\text{l}$ aliquot of each fraction. When $(\text{rCm})_n \cdot (\text{dG})_{12-18}$ was used as the template-primer, $4\text{ }\mu\text{g}$ of ^3H -dGTP (20 Ci/mM, 45,000 cpm/pmole) was used. At the end of the reaction, $90\text{ }\mu\text{l}$ samples were applied to glass fibre discs (Whatman, GF/C), which were washed 5 times in 5% TCA/1% $\text{Na}_4\text{P}_2\text{O}_7$ and twice in ethanol, dried and counted in a Liquid Scintillation Spectrometer using a toluene-base scintillation fluid. DNA-directed DNA polymerase and terminal deoxynucleotidyl transferase activities were assayed using $2\text{ }\mu\text{g}$ of $(\text{dA})_n \cdot (\text{dT})_{12-18}$ and $2\text{ }\mu\text{g}$ of $(\text{dA})_{12-18}$ as the template-primer, respectively.

RESULTS

Phosphocellulose Column Chromatography of RDDP Activity

The particulate fraction obtained by centrifuging the culture medium was resuspended and applied to sucrose density gradients. A sharp peak of RDDP activity was observed at a density of $1.15\text{--}1.17\text{ g/cm}^3$ which corresponds to that of retrovirus particles.²³ This RDDP activity was detected on day 3 and persisted

Fig. 1 Phosphocellulose column chromatography of RNA-directed DNA polymerase activity. ^3H -dTTP used as the labelled substrate for $(\text{rA})_n \cdot (\text{dT})_{12-18}$. a, b, c, B cell stimulated thymus cell (culture medium). d, MuLV producer cells. e, fresh thymus cells. f, B cell (Raji). g, PHA-stimulated thymus cells. h, mitomycin C treated B cells (culture medium).

until day 7. Sucrose density gradient fractions containing RDDP activity were pooled and subjected to chromatography on phosphocellulose. Extracts of thymus cells and B cells were used as controls. The culture media of MuLV (murine leukemia virus) producer cell line, PHA-stimulated thymus cells, and mitomycin C-pretreated B cells were also examined. RDDP activity in the culture medium from MuLV producer cell lines was eluted at 0.17 M KCl (Fig. 1-d).²⁶ RDDP activity in the culture medium of B cell stimulated thymus cells was also eluted at 0.17 M KCl (Fig. 1-a, b, c). Essentially, no RDDP activity was eluted at 0.17 M KCl when the other samples were chromatographed; the RDDP activity in

Table 1
Template-primer requirement of RNA-directed DNA polymerase

The template-primer specificity of RDDP activity in B cell stimulated thymus cells was examined. Assays were carried out for 30 min in the reaction mixture described in Materials and Methods. The template-prime concentration was 20 μ g. ³H-dTTP was used as the labelled substrate for (rA)_n·(dT)₁₂₋₁₈ and (dA)_n·(dT)₁₂₋₁₈, and ³H-dGTP for (rCm)_n·(dG)₁₂₋₁₈ and (dA)₁₂₋₁₈. Numbers in parentheses represent the activity in the presence of Mg⁺⁺ (10 mM) instead of Mn⁺⁺ (1 mM) as divalent cation.

Source of protein	³ H-dTMP or ³ H-dGTP incorporation (cpm)			
	(rA) _n (dT) ₂₋₁₈	(dA) _n (dT) ₁₂₋₁₈	(rCm) _n (dG) ₁₂₋₁₈	(dA) ₁₂₋₁₈
B cell stimulated thymus cell ^a				
0.17 M KCl phosphocellulose eluate	499 (186)	33	3780 (563)	0
Fresh thymus cell extract				
0.06 M KCl phosphocellulose eluate	12598	13450	494	558
0.38 M KCl phosphocellulose eluate	18860	15530	0	98
Fresh B cell (Raji) extract				
0.06 M KCl phosphocellulose eluate	21758	25528	0	N.D. ^b
0.35 M KCl phosphocellulose eluate	24208	13672	0	N.D.
Mitomycin C-pretreated B cell (Raji) ^a				
0.06 M KCl phosphocellulose eluate	544	2202	0	N.D.
0.15 M KCl phosphocellulose eluate	280	7220	0	N.D.
0.37 M KCl phosphocellulose eluate	850	604	0	N.D.

^a culture medium, ^b N.D.; not done

extracts from fresh thymus cells, B cells and PHA-stimulated thymus cells was not eluted at 0.17 M KCl, but at 0.06 M and 0.35 M KCl (Fig. 1-e, f, g). RDDP activity in the culture medium from mitomycin C-treated B cells was eluted at 0.15 M KCl, which is close to 0.17 M (Fig. 1-h). However, this was accompanied by a very high activity of DNA-directed DNA polymerase and probably represented EB virus-associated DNA polymerase²⁷ (see Table 1).

Template-Primer Specificity of RDDP Activity

The RDDP activity in B cell-stimulated thymus cells utilized $(rA)_n \cdot (dT)_{12-18}$ more efficiently than $(dA)_n \cdot (dT)_{12-18}$ as the template-primer (Table 1). It also utilized $(rCm)_n \cdot (dG)_{12-18}$.²⁸ This template-primer specificity is quite similar to that of other mammalian reverse transcriptases.²⁹ As for fresh thymus cells and B cells, the two peaks of RDDP activity that eluted at 0.06 M and 0.35 M KCl transcribed $(rA)_n \cdot (dT)_{12-18}$. However, almost the same or a higher level of DNA-directed DNA polymerase activity was observed, suggesting that these activities were due to contaminating cellular polymerases. The eluate at 0.15 M KCl in the medium of mitomycin C-treated B cells (see above) transcribed $(dA)_n \cdot (dT)_{12-18}$, but did not transcribe $(rCm)_n \cdot (dG)_{12-18}$. Eluates at 0.06 M KCl from both fresh and B cell-stimulated thymus cells transcribed $(rCm)_n \cdot (dG)_{12-18}$ very poorly.

DISCUSSION

The presence of reverse transcriptase in normal human tissues has thus far been not conclusive. Positive results have been reported in placenta, sperm, milk, and the cultured fibroblasts.^{11,12,30-32} On the other hand, Green *et al.* could not detect the particle-associated reverse transcriptase activity in a variety of normal human cells, of both adult and embryonic origin.³³ In our study reverse transcriptase activity could be demonstrated in the culture medium of B cell-stimulated fetal thymus cells. The enzyme activity differed from cellular polymerases in terms of template-primer requirement and ionic strength at which it was eluted from phosphocellulose. In an accompanying paper,³⁴ we have detected retrovirus-like particles in fetal thymus cells by electron microscopy. RDDP activity sedimented in sucrose density gradients at the density characteristic of animal retrovirus. These RDDP activities detected here are therefore most probably reverse transcriptases.

Some authors have reported that B cell lines are contaminated with retroviruses.³⁵ Since reverse transcriptase activities and retrovirus-like particles were also present after stimulation with peripheral B cells, as well as B cell lines,³⁴ retroviruses are unlikely to have been introduced by B cell lines.

To our knowledge, this is the first demonstration of reverse transcriptase activities in freshly isolated human thymus cells by induction. Our results that reverse transcriptase activities were not present in fresh fetal thymus cells but only after induction suggest that RNA tumor provirus activation in human fetal thymus cells. Further characterization of the reverse transcriptase activities is needed for detailed information concerning the significance of retrovirus in human thymus.

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