Oncornavirus-like particles from cultured bone marrow cells preceding leukemia and malignant histiocytosis

(human leukemia/preleukemia/reverse transcriptase)

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ABSTRACT Particles with the density and enzymatic activity characteristic of known oncornavirus have been previously described in bone marrow cells from patients with leukemia in relapse and in remission. We have confirmed these findings and studied two patients in whom preleukemia was among the diagnostic considerations. Following cultivation of bone marrow from these patients for 1 week in conditioned media with dexamethasone, a high-speed pellet of the supernatant fluid and disrupted cells was prepared and analyzed on a sucrose gradient for enzymatic activity characteristic of RNA-directed DNA polymerase (reverse transcriptase). Peaks of endogenous DNA polymerase activity showing ribonuclease sensitivity and/or stimulation with the synthetic template poly(rC)(dG)₁₂₋₁₈ were demonstrated in both patients at densities of 1.15 to 1.19 and 1.21 to 1.24 g/ml. Subsequently, diagnosis 2 and 4 months after initial evaluation revealed acute myelogenous leukemia and malignant histiocytosis, respectively. Prior studies have suggested a possible etiological significance of such particles in human leukemia. The demonstration of similar particles preceding clinically overt disease in these patients supports this hypothesis and offers the possibility of early diagnosis and treatment.

A cytoplasmic particle with the density, high-molecular-weight RNA, and RNA-dependent DNA polymerase characteristic of known RNA tumor viruses has been demonstrated in malignant cells from patients with leukemia (1-3). Following cultivation with conditioned media, Mak *et al.* have demonstrated an increase and release of such particles from cells of leukemic patients both in relapse and in firm hematological remission with no evidence of disease (3, 4). Molecular hybridization (5-7) and immunological studies (8, 9) have added significance to these particles by demonstrating a close relationship between them and known oncogenic agents of primate origin, confirming earlier reports of homology between nucleic acid of human leukemia cells and murine leukemia virus (10, 11).

We now report studies of two patients in whom, on the basis of history and clinical and laboratory examination, the possibility of preleukemia was considered as part of the differential diagnosis. Extensive investigation of both patients failed to reveal any definite histological evidence to support the diagnosis of leukemia. Bone marrow obtained at that time from both patients showed clear biochemical evidence of oncornavirus-like particles 2 and 4 months prior to the diagnosis of acute myelogenous leukemia and malignant histiocytosis, respectively.

Abbreviations: AML, acute myelogenous leukemia; CML chronic myelogenous leukemia.

MATERIALS AND METHODS

Patient Material. Bone marrow from these two patients and from patients with either acute myelogenous leukemia (AML) or chronic myelogenous leukemia (CML) was obtained by aspiration from the posterior iliac crest. Peripheral blood was used in cases with high peripheral blast counts. Normal bone marrow was obtained from three normal marrow donors in the course of bone marrow transplantation.

Method of Cultivation. Bone marrow or peripheral blood cells were obtained and the buffy coat prepared for culture. Cells were cultured, as described by Mak *et al.* (3) with slight modification, at 10⁶ cells/ml in McCoy's 5A modified media containing 15% (v/v) fetal calf serum, 10% conditioned media from human embryo kidney cells, 5 µg/ml of dexamethasone, and antibiotics. Cultivation was done in 10 ml Falcon plastic tubes containing 5 ml each of the cell suspension. Typically, 10⁸ cells were used in an experiment. The fetal calf serum and conditioned media were assayed for polymerase activity and shown to be negative prior to use.

Preparation of Pellet. After cultivation for 7-9 days, the supernatant fluid was harvested and clarified at 10,000 × *g* for 15 min at 4°. The cells were resuspended in 5 ml of 5% sucrose, 0.001 M EDTA, 0.01 M Tris-HCl at pH 8.1, and ruptured with 10 strokes of a Dounce homogenizer. After clarification at 10,000 × *g* for 15 min at 4°, the supernatant from the disrupted cells was added to the clarified culture media and pelleted through a 20% glycerol pad in a SW27 rotor in a Beckman L2 centrifuge at 25,000 rpm for 90 min at 4°. The combined high-speed pellet was resuspended with 0.5 ml of 5% sucrose in TNE buffer (Tris-HCl 0.01 M at pH 8.1, NaCl 0.1 M, EDTA 0.001 M), layered onto a 20-60% sucrose gradient in TNE and spun for 16 hr in a SW41 rotor at 30,000 rpm. Fractions of 0.5 ml were collected and assayed in triplicate for ribonuclease-sensitive endogenous DNA polymerase and/or poly(rC)-(dG)₁₂₋₁₈-stimulated DNA polymerase activity.

Conditions of Assay. Each assay consisted of 40 µl of gradient material in a final volume of 100 µl containing 50 mM Tris-HCl at pH 8.1, 40 mM NaCl, 6 mM MgCl₂, 0.1% Triton X-100, 5 mM dithiothreitol, 1 mM each dATP, dCTP, dTTP and 0.45 nM [³H]deoxyguanosine triphosphate (specific activity 22 Ci/mmol). Reactions were run for 1 hr at 37°. Poly(rC)-(dG)₁₂₋₁₈ (Collaborative Research) was used at a final concentration of 1.0 A₂₆₀ unit/ml.

Reactions were terminated with the addition of 100 µg of yeast RNA and 2 ml of 10% trichloroacetic acid containing

Table 1. DNA polymerase activity from bone marrow cultures (cpm/10⁶ cells)*

Patient	Diagnosis	Density					
		1.15–1.19 g/ml			1.21–1.25 g/ml		
		RNase sensitive	Endogenous	(rC) _n ·(dG) ₁₂₋₁₈ stimulated	RNase sensitive	Endogenous	(rC) _n ·(dG) ₁₂₋₁₈ stimulated
D.C. I		0	162	4804	0	45	4779
R.M.		405	850	NT	42	372	NT
D.C. II	AML	NT	661	577	NT	152	411
R.H.	AML	NT	449	1659	NT	349	2014
I.B.	CML	NT	9	514	NT	8	418
D.Z.	CML	425	2613	6407	234	1502	4845

D.C. is the patient who subsequently developed acute myelogenous leukemia. R.M. is the patient who subsequently developed malignant histiocytosis. NT, not tested; AML, acute myelogenous leukemia; CML, chronic myelogenous leukemia.

* Background approximately 200 cpm is subtracted; values represent sum of cpm under each peak.

1% sodium pyrophosphate. After precipitation at 0° for 20 min, the product was collected on a Gelman type E glass fiber filter and washed with 40 ml of 5% trichloroacetic acid. Filters were dried and the radioactivity counted in a Tricarb scintillation counter with toluene and BBOT [2,5-bis-2-(5-*tert*-butylbenzoxazolyl)thiophene] (4 g/liter).

Case History No. 1. D.C. is a 13-year-old boy who was well until August of 1974 when he presented with a 3 month history of bruising easily, and recent upper respiratory tract infection with a temperature of 104°. Physical examination showed multiple ecchymoses, tonsillar exudates, and shotty adenopathy. Laboratory examination revealed a hemoglobin of 10.9 g/ml, white blood count of 4500/mm³ with a normal differential, and platelets of 38,000/mm³.

Throat culture showed β -hemolytic streptococci, non-group A; other routine bacterial and viral cultures were negative. Heterophile titer was negative. The sedimentation rate was 155 mm/hr. The serum vitamin B12 and B12-binding capacity were elevated to 1436 pg/ml and 1978 pg/ml, respectively (normal <1000 pg/ml). Leukocyte alkaline phosphatase and fetal hemoglobin were normal.

Investigation for autoimmune disease was negative. Initial bone marrow examination was interpreted as moderately hypercellular with erythroid and myeloid hyperplasia and abundant megakaryocytes with no morphological evidence of malignancy. Over the next several days, the patient developed severe erythema nodosum, hepatosplenomegaly, progressive leukopenia, thrombocytopenia, and anemia requiring transfusion. On October 10, 1974, he was begun on 75 mg of prednisone daily with prompt lysis of his fever. His anemia, leukopenia, and thrombocytopenia improved rapidly over a 2-week period.

A total of four bone marrow examinations, done between 9/4/74 and 10/25/74, were hypercellular with no evidence of malignancy. Examination of bone marrow obtained on 10/8/74 showed evidence of oncornavirus-like particles (Fig. 2A). The patient was followed in the outpatient clinic where a bone marrow 2 months later (12/12/74) revealed a total of 4% myeloblasts, most of which had Auer rods. Examination of a bone marrow on 2/7/75 showed 38% myeloblasts and again revealed evidence of oncornavirus-like particles (Fig. 2B). His subsequent clinical course has been typical for a patient with AML, with several remissions and relapses.

Case History No. 2. R.M. was noted since 6 months of age to have recurrent upper respiratory tract infections and otitis media. Evaluation in March of 1974 at the age of 2 years re-

vealed a history of bruising easily and hepatosplenomegaly on physical examination. White blood count was 26,500/mm³ and platelet count was 33,000/mm³. Serum vitamin B12 was elevated to 1900 pg/ml. Fetal hemoglobin was slightly elevated to 4.1% of total hemoglobin. Leukocyte alkaline phosphatase was normal. The bone marrow showed increased mature cells of the granulocytic series with a paucity of megakaryocytes and erythrocyte precursors. A liver biopsy in April 1974 was normal. In July of 1974, the patient developed a coagulopathy with gastrointestinal hemorrhage. Platelets ranged between 2,000 and 100,000/mm³. The bone marrow was hyperplastic with increased erythroid, megakaryocytic, and histiocytic components with no morphological evidence of malignancy. Immunological evaluation was normal. A splenectomy, liver biopsy, and lymph node biopsy showed plasma cell and histiocytic infiltration consistent with an atypical lymphoproliferative disorder. Bone marrow examination in August 1974 showed granulocytic and megakaryocytic hyperplasia, but no evidence of malignancy. Examination of this marrow after culture revealed oncornavirus-like particles (Fig. 3).

A definitive diagnosis of malignancy could not be made. However, the patient, on the basis of his clinical picture and laboratory examination, was begun on cyclophosphamide, vinblastine, and steroids. Despite this treatment, the patient expired in December of 1974. Autopsy revealed malignant histiocytosis (leukemic reticuloendotheliosis) involving the bone marrow, liver, lymph nodes, heart, and pericardium (12).

RESULTS

Three untreated patients with AML or CML showed evidence of particles banding at densities of 1.15–1.19 and 1.21–1.24 g/ml with poly(rC)·(dG)₁₂₋₁₈-stimulated polymerase activity (Fig. 1, Table 1) confirming previous results of Mak *et al.* (4). Identical results were obtained in patient D.C., both before (Fig. 2A) and after (Fig. 2B) the diagnosis of AML. In patient R.M., ribonuclease-sensitive DNA polymerase, present prior to his demise and diagnosis, showed peaks of activity at densities of 1.16 and 1.24 g/ml (Fig. 3). Poly(rC)·(dG)₁₂₋₁₈-stimulated activity was not determined in this experiment. The high-speed pellet of the supernatant fluid and disrupted cells were pooled in all experiments to obtain maximal sensitivity. Thus, we cannot ascertain from these studies if there was release of particles into the media.

FIG. 1. RNase sensitive, endogenous and poly(rC)-(dG)₁₂₋₁₈-stimulated DNA polymerase activity from human leukemia cells. After culture, a high-speed pellet of supernatant fluid and disrupted cells from a patient (R.H.) with AML was spun to equilibrium in a 10–60% sucrose gradient and assayed for: □, RNase-sensitive DNA polymerase; ○, endogenous DNA polymerase; and Δ, poly(rC)-(dG)₁₂₋₁₈-stimulated DNA polymerase.

Similar assays conducted on bone marrow obtained from three absolutely normal bone marrow donors were negative (data not shown).

DISCUSSION

Oncornavirus-like particles and reverse transcriptase have previously been described in human leukemia cells in studies in which several different techniques were used (1–4). This work confirms prior observations and extends them to two patients with preleukemia.

FIG. 2A and B. Bone marrow cells from patient D.C. were cultured and assayed as described for □, RNase sensitive-DNA polymerase activity; ○, endogenous DNA polymerase; and Δ, poly(rC)-(dG)₁₂₋₁₈-stimulated activity. (A) results from marrow obtained prior to overt disease; (B) results obtained after diagnosis of AML.

FIG. 3. Bone marrow cells were obtained from patient R.M. and analyzed as described for □, RNase-sensitive DNA polymerase; and ○, endogenous DNA polymerase activity. Four months later the patient expired and was found to have malignant histiocytosis.

The particles isolated from bone marrow cells of these patients are, by two criteria (density and enzymatic activity), identical to known oncornavirus. A density of 1.15–1.19 g/ml is characteristic of complete viral particles (13) whereas, the density 1.21–1.25 g/ml has been associated with viral cores from murine leukemia (14, 15) and with cores of virus-like particles from human breast milk (16). Previous studies have shown the ability to utilize the synthetic template poly(rC)-(dG)₁₂₋₁₈ to be a characteristic of reverse transcriptase; this is not seen with normal cellular polymerases (9, 17, 18). The present experiments do not exclude the presence of a terminal transferase (16). Recent reports describe this enzyme in normal bone marrow, AML and CML in blast crises (19, 20). However, techniques similar to those used here have failed to detect this enzyme in the virus-like particles isolated from the cells of patients with AML, or CML (3).

Treatment with drugs, a number of specific congenital syndromes, and a variety of acquired hematological changes, most frequently refractory macrocytic anemia, have been associated with the subsequent development of leukemia in man (21, 22). In a recent report of 123 patients with leukemia, 31% had a documented preleukemic phase (23).

The close relationship between leukemia and preleukemia has been supported by various laboratory abnormalities common to both. An elevated serum vitamin B12 concentration, observed in both of our patients, is frequently present in chronic myelogenous leukemia (24). Patient R.M. had a mildly elevated fetal hemoglobin previously noted in patients with leukemia (25). Other common abnormalities include chromosome changes (26, 27), and defective *in vitro* bone marrow maturation (28, 29). The present study identifies another characteristic, oncornavirus-like particles that are common to leukemia and preleukemia.

Nonleukemic hematological abnormalities preceding leukemia are not peculiar to man and have been observed both in the natural history of feline leukemia (30) and after laboratory infection with murine (31) and feline leukemia virus (32). Thus, it is not surprising and, indeed, would be expected, that a putative viral agent associated with leukemia in man should also be present in the preleukemic phase. In addition, the presence of particles preceding overt human disease argues against the possibility that these particles are

merely a consequence of the presence of malignancy. Recently, long term virus production by human leukemia cells in culture has been reported; this suggests that formation of an infectious particle is possible and might occur naturally (33). Preleukemia could represent the early expression of such an agent.

We have not found similar particles in three absolutely normal controls. Mak *et al.*, using the same technique, have found evidence of similar particles in one patient with diabetes and anemia among a total of 13 patients with nonmalignant diseases (3, 4, 7). Additional studies may reveal an association of such particles in man with nonmalignant disease, as observed in the feline (28).

The demonstration of oncornavirus-like particles in patients with clinical and laboratory evidence of preleukemia is consistent with and may be diagnostic of eventual leukemia. Molecular hybridization is yet needed to show that, indeed, these particles from preleukemic patients contain leukemogenic information as has been demonstrated in similar particles from leukemic patients (6, 7). Should this be true, diagnosis and treatment in the preleukemic phase of the disease may be feasible.

Obviously, further studies of additional normal controls, patients suspected of having preleukemia, and a variety of patients with nonmalignant disease are needed.

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