Type C RNA Tumor Virus Isolated from Cultured Human Acute Myelogenous Leukemia Cells

Abstract. *Previously, type C RNA tumor virus-related components have been described in blood leukocytes from patients with acute myelogenous leukemia. These components, for example, reverse transcriptase, have been shown to be most closely related to those from two oncogenic subhuman primate type C viruses (woolly monkey sarcoma virus and gibbon ape leukemia virus). Now, we report the continuous production of budding type C viruses with the same characteristic reverse transcriptase by three separate culturings of leukocytes from a single bleeding from a patient with acute myelogenous leukemia. These isolations were made possible by the discovery of a source of conditioned media which sustains exponential growth of human myelogenous leukemia cells in liquid suspension culture.*

RNA tumor viruses, which are classified morphologically as type C (*1*), have been associated with the development of leukemia, lymphoma, and sarcoma in several vertebrates (*2*). These findings have recently been extended to subhuman primates. First, a type C virus was isolated from a naturally occurring fibrosarcoma in a New World primate, the woolly monkey (*3*). It was subsequently shown to produce lymphosarcomas on injection into secondary primate hosts (*4*). Second, type C viruses were isolated from naturally occurring lymphosarcomas and myelogenous leukemias of an Old World primate, the gibbon ape (*5, 6*), a species phylogenetically closely related to man. In several instances, cell-free extracts of gibbon ape leukemia cells have produced viremia or myelogenous leukemia (or both) in juvenile gibbon ape recipients (*6, 7*). These observations provide reasons to suspect that similar viruses may be involved in leukemogenesis in man. However, extensive investigations of human leukemic materials by electron microscopy and by classical virological infectivity studies have so far failed to provide convincing evidence for the presence of complete type C viruses in human leukemia cells (*2*).

In 1970, shortly after the discovery of RNA-directed DNA polymerase (reverse transcriptase) in RNA tumor viruses (*8*), our laboratory demonstrated a similar RNA-directed DNA polymerase activity in the particulate cytoplasmic fraction of fresh peripheral blood leukocytes from three patients with acute leukemia (*9*). Subsequently, we showed that this enzyme had biochemical properties indistinguishable from reverse transcriptase from mammalian type C viruses (*10*). With human acute myelogenous leukemia cells, this polymerase was shown by immunological studies to be specifically related to reverse transcriptase from the two known oncogenic primate type C viruses, the woolly monkey (simian) sarcoma virus (SiSV) and the gibbon ape leukemia virus (GALV) (*11–14*). Also, nucleic acids associated with the reverse transcriptase have some nucleotide sequences that are similar to those of some RNA tumor viruses (*14–17*). Finally, fresh blood leukocytes from five cases of acute myelogenous leukemia tested were recently determined to contain a protein strongly related to group specific antigen (p30 protein) from SiSV (*18*).

These studies then indicate that some proteins related to components of type C viruses are already expressed in many cases of human acute myelogenous leukemia. Moreover, in some cases, they are apparently associated intracellularly in a particulate form, which has some of the biophysical properties of a complete RNA tumor virus (*14, 15, 17*). In this regard, the transient release of particles from human leukemic cells containing "reverse transcriptase" has been reported (*19*) after short-term culture under select conditions. However, without continuous production of virus, without the demonstration of virus budding from cell membranes (*1*), and without definitive characterizations of viral components, it is not possible to conclude that these particles indicate the isolation of an RNA tumor virus. These particles are probably related to the intracellular, presumably defective, particles which we have previously described in human leukemia cells (*14, 15*).

We now report the sustained release of typical, budding type C virus from several cultures of peripheral blood leukocytes from one patient (HL-23) with acute myelogenous leukemia (AML). Moreover, the virus contains reverse transcriptase, as reported here, and group specific antigen (*19a*) specifically related to those from the two oncogenic primate type C viruses, as would be anticipated from our previous studies with the fresh myelogenous leukemia cells of this patient (*13*) and of other patients (*11, 12, 14*).

We were able to isolate this type C virus only because we recently discovered (and used) a factor which is released by certain growing cells into their culture medium and which supports the continued exponential growth and myeloid differentiation in liquid suspension culture of leukemic myeloid leukocytes (*20*). The properties of the culture system include: (i) dependence for leukocyte growth and differentiation on a factor produced by a particular culture strain of whole human embryo cells (WHE-1); (ii) growth of myeloid cells from the peripheral blood or bone marrow of patients with acute or chronic myelogenous leukemia but not of blood or marrow cells from normal donors or patients with lymphatic leukemia; (iii) increase in cell number, 10- to 100-fold during 8 to 10 days culture after an initial 3- to 4-day lag period of growth; (iv) maintenance of the same growth kinetics over at least 4 months, permitting the accumulation of gram quantities of leukocytes from an initial cell seeding of 2×10^6 cells; (v) maintenance of approximately the same differential leukocyte count during the period of culture, except for a decline in the proportion of lymphoid cells; and (vi) identification of a marker chromosome in all metaphases of cultured myeloid leukocytes from two patients, indicating the growth in suspension culture of the leukemic cell clone.

The present patient (HL-23), a 61-year-old woman, was admitted to M. D. Anderson Hospital, Houston, Texas, with a 2-month history of tiredness and pallor. There was no family history of leukemia or lymphoma, no antecedent medical condition associated with the development of leukemia, and no known exposure to animals with tumors or blood diseases or exposure to physical or chemical agents associated with the development of leukemia (*14, 21*). However, she did have contact prior to the onset of the disease with a friend who had leukemia. The peripheral white blood cell count was increased to 125,000 cell/mm^3, and on the basis of cytological examinations of the peripheral blood and bone mar-

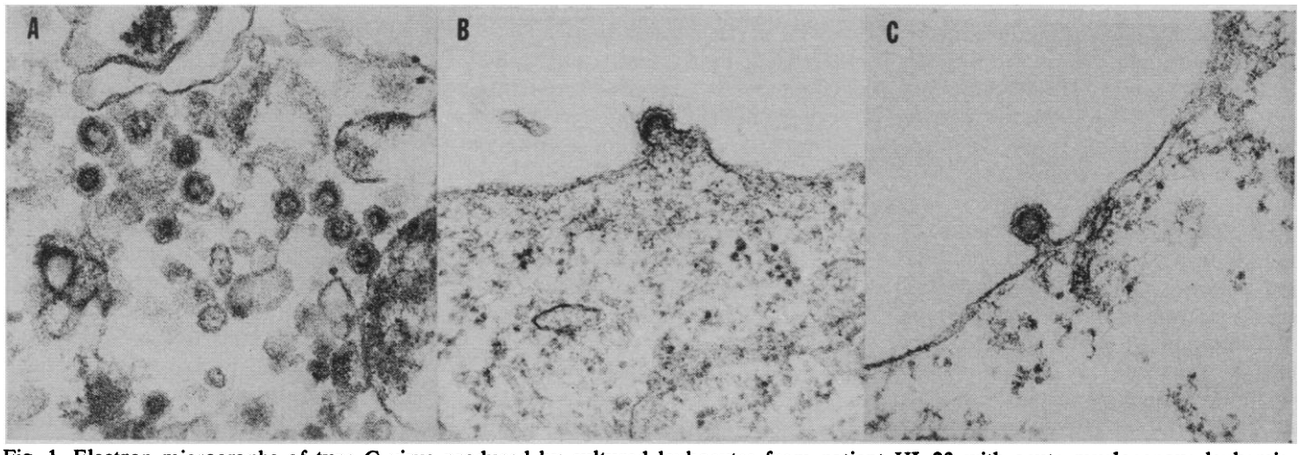

Fig. 1. Electron micrographs of type C virus produced by cultured leukocytes from patient HL-23 with acute myelogenous leukemia. Tissue culture fluid was processed, and the media pellet was isopycnically banded on a sucrose gradient (*12*). Fractions were collected, and DNA polymerase was assayed in a final reaction volume of 0.05 ml containing 50 m*M* tris, *p*H 7.8, 100 m*M* KCl, 0.5 m*M* $MnCl_2$, 20 m*M* dithiothreitol, 0.05 percent Triton X-100, bovine serum albumin (1 mg/ml), 16 μM [^{3}H]thymidine triphosphate (dTTP) (New England Nuclear, 6582 count/min per picomole), 5 μl of enzyme and oligodeoxythymidylate · polyadenylate (40 μg/ml) [oligo(dT) · poly(A); Collaborative Research, Waltham, Mass.]. Reaction mixtures were held for 30 minutes at 37°C. A peak of activity, incorporating 4 pmole of [^{3}H]thymidine monophosphate per hour per 5 μl was detected at a density of 1.16 g/ml. This peak was processed for thin-sectioning (*30*) and examined with a Hitachi HU 11E electron microscope. (A) Free type C virus particles from the sucrose gradient banded culture fluid; (B) an early, budding virus; (C) completed viral bud (× 120,000).

row, which showed 70 to 80 percent myeloblasts, the diagnosis was made of acute myelogenous leukemia.

Prior to any therapy, white blood cells were withdrawn from the patient with an IBM blood cell separator (*22*). More than 95 percent of the leukocytes were viable, as determined by trypan blue dye exclusion. The leukemic leukocytes were processed the same day in a biohazard safety cabinet (Bioquest, Cockeysville, Md.), which had been irradiated with ultraviolet light for a minimum of 15 minutes before use. Sterile, disposable pipettes, tubes, and flasks (Falcon) were used in all procedures. The cells were washed twice in phosphate-buffered saline, *p*H 7.2. A portion of the saline-washed blood cells was used directly for biochemical analysis. As was previously reported with the fresh blood leukocytes of this patient (*13*), we found reverse transcriptase immunologically related to reverse transcriptase from the woolly monkey sarcoma virus in the cytoplasmic microsomal membrane fraction. A portion of the fresh whole cells and of the microsomal membrane fraction of homogenized cells was fixed in 3 percent glutaraldehyde. An extensive search by electron microscopy of thin sections of these materials failed to reveal virus particles (*23*).

For tissue culture, the leukemic blood leukocytes were suspended in RPMI 1640 medium, containing 20 percent fetal calf serum (Reheis Corp., Phoenix, Ariz.; serum lot L13203). One leukocyte culture strain (I-1) was derived directly from these fresh blood leukocytes by cocultivating with a monolayer culture of WHE-1 cells. After 1 week of cocultivation, the suspension culture cells were removed and, thereafter, growth was sustained by adding filtered 10 percent WHE-1 conditioned media to the suspension culture system. A second leukocyte culture strain (I-2) was derived at a later date from a portion of the I-1 cultured leukocytes that were frozen at the second tissue culture passage. A third leukocyte strain (II-1) was derived from a separate frozen portion of the previously uncultured fresh blood leukocytes of the patient and this strain was initiated entirely with filtered WHE-1 conditioned media rather than by cocultivation. The cultured leukocytes were maintained in a facility in which only nonvirus producing cell lines were carried. Sources of media, fetal calf serum, conditioned media, and WHE-1 cells were examined for possible viral particles by repeated electron microscopy, for reverse transcriptase by assays of DNA polymerase in the medium, and for infectivity by testing on numerous uninfected cell culture lines; all such tests were negative. Karyotype analysis of the cultured leukocytes as well as of the patient's bone marrow sample at diagnosis showed a typical female chromosome pattern. Some metaphases from both sources showed a possible abnormality of a G group chromosome.

Culture fluid samples from the leukocyte suspension cultures were periodically assayed for the release of particles containing reverse transcriptase. Positive results were obtained from all three strains of the cultured leukocytes, from strain I-1 at passage 10, from strain I-2 at passage 10, and from strain II-1 at passage 4. The DNA polymerase from the virus had properties characteristic of mammalian type C virus reverse transcriptase (*10, 14, 24*). In addition, the reverse transcriptase activity prior to its being extracted banded isopycnically on a sucrose gradient at a density of 1.16 g/ml, the density of banding for RNA tumor viruses.

Fractions banding at 1.16 g/ml were centrifuged at 98,000*g*, and electron micrographs were made from sections of the pelleted material. Numerous virus particles were observed in pellets; this result was obtained with each of the three leukocyte cultures from the same bleeding (Fig. 1A). Typical type C viruses were also detected budding from the cultured cells (Fig. 1, B and C).

An important consideration is whether the type C virus released by the cultured leukocytes contains components related to those found in the fresh AML blood cells. For this reason, we examined the effect of antiserums (IgG) to reverse transcriptase purified from SiSV on this enzyme from the virus derived from the human AML cells. As discussed above, we have previously

determined that such antiserums inhibit reverse transcriptase from fresh AML blood cells (*11, 12, 14*), including that from this patient (*13*). Antiserums to reverse transcriptase from SiSV inhibited this enzyme from either SiSV or GALV to a similar degree (Fig. 2A), confirming previous reports of the immunological relatedness of these viral enzymes (*11, 25*). Similarly, reverse transcriptase from either the type C virus produced by the cultured leukocytes (Fig. 2A) or from the fresh uncultured blood leukocytes [Fig. 2B and (*13*)] was inhibited by antiserum to SiSV reverse transcriptase. On the other hand, the rat antiserum to SiSV did not inhibit reverse transcriptase from an unrelated, avian type C virus (Fig. 2A), nor did nonimmune serum obtained prior to immunization of the rat inhibit HL-23 viral or cellular (Fig. 2B) reverse transcriptase. The immunological relatedness of reverse transcriptase from the type C virus produced by the cultured human AML cells to reverse transcriptase from various other RNA tumor viruses has also been studied (*24*).

From our previous findings with reverse transcriptase from human AML cells (*14*), these immunological data provide results that would be anticipated by the incorporation of intracellular reverse transcriptase into a complete virus particle. Further, reverse transcriptase with the same immunological properties was identified in each of the three separate leukocyte cultures. These circumstances argue against the possibility of accidental virus contamination of the cultured leukocytes. For this to have occurred, one must postulate two or more separate superinfection events in tissue culture with the same viral agent that would have had to contain components closely related to those already present in the fresh uncultured AML cells. Even so, biochemical studies of the components of the putative human type C virus should be compared to those of known type C RNA tumor viruses, and further attempts to obtain further isolates of virus from the cells of this patient are necessary.

We think that the expression of a complete type C virus from the cultured leukocytes is related to the conditioned media factor (or factors) which promotes the sustained exponential growth and differentiation of the leukemic myeloid cells (*20*). This could be secondary to the activation of DNA synthesis, which has been shown to be important for initiating RNA tumor virus replication in the absence or presence of certain virus-inducing agents (*26*). Alternatively, it could be related to the differentiation stimulus imposed on the human leukemic cells in a fashion analogous to the stimulation of Friend murine leukemia virus production by factors that promote the differentiation of mouse erythroleukemia cells (*27*). We have not yet identified type C viruses in 20 cultured myeloid cell strains from 16 other patients with myelogenous leukemia (*28*), although we have not studied them as intensely as we studied HL-23 leukocyte cultures. This may be analogous to the infrequent isolation of virus particles from genetically disparate outbred populations of animals prior to the availability of inbred strains (*29*). In view of the evidence presented for expression and partial assembly of viral components in fresh human myelogenous leukemia and in view of the relatedness of these components to those from oncogenic primate type C viruses (*14*) in which there is evidence for horizontal spread, the occasional isolation of a type C

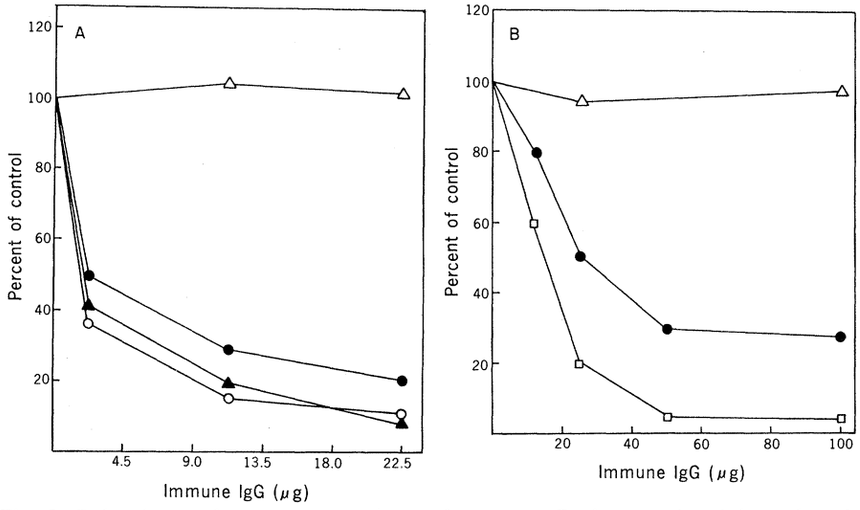

Fig. 2. Relatedness of reverse transcriptase from type C virus produced by cultured human myelogenous leukemia cells (HL-23) and from fresh leukemic blood leukocytes (HL-23) to reverse transcriptase from tumorigenic primate type C virus. The preparation of partially purified reverse transcriptase from the animal type C viruses (*31*) and from the fresh leukocytes of the patient (*13*) have been described. The procedures for preparing antiserums to DNA polymerases in rats and for purifying immune and nonimmune rat IgG (immunoglobulin G) have also been described (*32*). (A) Effect of immune IgG to reverse transcriptase from woolly monkey (simian) sarcoma virus (SiSV) on reverse transcriptase from HL-23 virus and from related (primate) and unrelated (avian) RNA tumor viruses. DNA polymerase was extracted from the HL-23 virus by incubating at 4°C for 15 minutes in 75 m*M* tris, *p*H 7.5, 0.5*M* KCl, 0.5 percent Triton X-100, 1.5 m*M* dithiothreitol and bovine serum albumin (20 mg/ml) (BSA). In a threefold dilution of this enzyme buffer, but containing 4 mg of BSA per milliliter, 5 μl of the various enzymes were incubated with 10 μl of IgG at 4°C for 2 hours. This mixture was then added to a reaction mixture as described in Fig. 1 for assay of polymerase activity with oligo(dT) · poly(A) and with [^{3}H]TTP (New England Nuclear; 16,232 count/min per picomole). The samples were incubated at 37°C for 60 minutes. Values are presented as the percentage of control DNA polymerase activity in the absence of immune IgG. No inhibition and less than 10 percent stimulation (compared to a buffer control) of DNA polymerase activity was observed in the presence of nonimmune serums. All antibody titrations were performed with a constant concentration of total IgG by compensating for the variable amount of immune IgG with an appropriate amount of similarly purified nonimmune IgG. Incorporation of tritium was 20,000 to 100,000 count/min in the absence of immune IgG. The sources of viral reverse transcriptase were: ●——●, type C virus from human cultured cells (HL-23); ○——○, woolly monkey sarcoma virus; ▲——▲, gibbon ape leukemia virus; and △——△, avian myeloblastosis virus. (B) Effect of rat IgG before (nonimmune) and after immunization with reverse transcriptase from woolly monkey type C virus (immune) on reverse transcriptase from the microsomal-membrane fraction of fresh leukemic leukocytes of patient HL-23. Assay conditions have been described (*13*) and are essentially the same as in (A) except that variable IgG concentrations were achieved by dilution with 0.1*M* tris, *p*H 8.0, and the preliminary incubation of enzyme and IgG was only for 10 minutes. ●——●, HL-23 AML cell enzyme with immune IgG; △——△, HL-23 AML cell enzyme with nonimmune IgG; and □——□, woolly monkey sarcoma virus enzyme with immune IgG.

virus from human leukemic cells under unusual culture conditions should perhaps not seem so surprising, despite many previous failures (*2*). Our results raise the possibility that in some patients with leukemia a complete virus particle could be assembled with the potential for horizontal transmission. Against such a mode of transmission, if the acquisition of disease is used as a measurement, is a good deal of epidemiological evidence (*14, 21*). This suggests that if virus can enter from without, interaction with other factors is required to produce myelogenous leukemia in man.

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6 January 1975

Polypeptide Composition of Fraction 1 Protein from Parasexual Hybrid Plants in the Genus Nicotiana

Abstract. *Analysis of the subunit polypeptide composition of Fraction 1 proteins gives information on the expression of both nuclear and chloroplast genomes; the large subunits of the protein are coded by chloroplast DNA, whereas the small subunits are coded by nuclear DNA. Fraction 1 protein isolated from the leaves of parasexual hybrid plants derived from the fusion of protoplasts of* Nicotiana glauca *and* N. langsdorffii *contains the small subunit polypeptides of both parent species and the large subunit polypeptides of only* N. glauca. *Fraction 1 protein isolated from the leaves of a hybrid plant obtained after the uptake of chloroplasts of* N. suaveolens *by protoplasts of white tissue of a variegating mutant of* N. tabacum *contains the large subunit polypeptides of both* N. suaveolens *and* N. tabacum, *as well as the small subunit polypeptides of both these species.*

By the fusion of protoplasts derived from mesophyll cells of leaves of *Nicotiana glauca* and *N. langsdorffii*, a parasexual hybrid plant was developed (*1*); it was self-fertile in contrast to conventional, reciprocal F_1 hybrids of *N. glauca* × *N. langsdorffii*, which are not self-fertile. Also, plants of a cytoplasmic hybrid character were derived after the incorporation of isolated chloroplasts of several different *Nicotiana* species into protoplasts obtained from the white tissue of a variegating mutant of *N. tabacum* (*2*).

The expression of both nuclear and chloroplast genomes in these parasexual hybrid plants may be examined by an analysis of the polypeptide composition of a single protein, the Fraction 1 protein. Fraction 1 protein (ribulose diphosphate carboxylase) consists of two kinds of subunits, which differ in size. The large subunit is coded by chloroplast DNA (*3*), whereas the small subunit is coded by nuclear DNA (*4*). By isoelectric focusing of Fraction 1 proteins of *Nicotiana* species in 8*M* urea, it has recently proved possible to resolve, in a single polyacrylamide gel, the large subunit into three polypeptides, and to resolve the small subunit into one or more polypeptides (*5*). The patterns obtained are reproducible and characteristic of each species and thus readily provide phenotypic markers for both chloroplast and nuclear DNA. The three large subunit polypeptides are always inherited and expressed together, with no separation of the inheritance of the individual polypeptides (*6*).

Plants of the parasexual hybrid of *N. glauca* and *N. langsdorffii* were grown from seed obtained from progeny of the original hybrid plant (*1*). The off-