Corrections

Correction. In the article "Growth and differentiation in culture of leukemic leukocytes from a patient with acute myelogenous leukemia and re-identification of type-C virus" by R. E. Gallagher, S. Z. Salahuddin, W. T. Hall, K. B. McCredie, and R. C. Gallo, which appeared in the October 1975 issue of Proc. Nat. Acad. Sci. USA 72, 4137–4141, the authors have requested the following changes. On p. 4140, left-hand column, second line from bottom, references 21–27 should be 19, 21–25. On p. 4140, right-hand column, lines 27 and 51, the initial reference in the cited series should be 26 instead of 23; in line 54 the two inclusive series of references should be 26–28 and 28–30 instead of 24–28 and 23–30, respectively. On p. 4141, left-hand column, line 10, the proper reference is 26, not 23.

Correction. In the article "Methylation of a membrane protein involved in bacterial chemotaxis" by E. N. Kort, M. F. Goy, S. H. Larsen, and J. Adler, which appeared in the October 1975 issue of Proc. Nat. Acad. Sci. USA 72, 3939–3943, the authors have requested the following changes. On page 3940, Table 1, the following data should have been inserted: e25p3, a cheB smooth mutant, shows 5% of wild-type methylation; AW620 and AW627, cheB tumbling mutants (see legend), show, respectively, 52 and 74% of wild-type methylation. The authors wish to add that radioactivity from 35S-labeled methionine is not incorporated into methyl-accepting chemotaxis protein (MCP); this provides additional evidence that the reaction is a methylation.

Correction. In the article "Transposition of R factor genes to bacteriophage λ" by Douglas E. Berg, Julian Davies, Bernard Allet, and Jean-David Rochaix, which appeared in the September 1975 issue of Proc. Nat. Acad. Sci. USA 72, 3628–3632, the authors have requested the following change. On page 3629 in Table 1, the heading of the third column of part B, which reads "Proportion KanR transducing phage," should read "Proportion lysogens among transductants."
Growth and differentiation in culture of leukemic leukocytes from a patient with acute myelogenous leukemia and re-identification of type-C virus

(liquid suspension culture/leukocyte growth-stimulating factor/granulocyte differentiation/chromosome abnormality/RNA tumor virus)

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ABSTRACT Conditioned medium from a culture of whole human embryo cells stimulated prolonged exponential growth in suspension culture of leukocytes from a patient with acute myelogenous leukemia. Ten to 20% of the cultured cells consistently differentiated into mature granulocytes including neutrophils, eosinophils, and basophils. The proportion of lymphocytes declined after culturing, and tests for Epstein-Barr virus antigens were negative. An abnormality of a G group chromosome was observed in some metaphases from the patient's fresh bone marrow and from the cultured leukocytes, indicating growth in vitro of leukemic cells. After 4–10 weeks in culture, a budding type-C virus was continuously released by the cultured leukocytes, predominantly by undifferentiated blast cells. This virus was originally identified in three different cultures of a peripheral blood specimen obtained at the time of diagnosis. Subsequently, this virus was identified by reverse transcriptase (RNA-dependent DNA polymerase) assays and by electron microscopy in cultured leukocytes from a bone marrow specimen obtained 14 months later from the same patient. Virus produced by cultures of both specimens was closely related, if not identical, to the woolly monkey type-C virus.

Attempts to sustain growth of human leukemic myeloid cells in tissue culture for more than 1–2 weeks have had few exceptions either failed or resulted in the growth of lymphoblastoid cells with no established relationship to possible leukemic cell progenitors (1). Most of the reported exceptions cite rather limited leukocyte growth for a few weeks, in some cases with evidence of myeloid differentiation (2–5). In two instances, myeloblastoid cell lines have been established in liquid suspension culture from a patient with acute myelogenous leukemia (AML) (6) and from a patient with chronic myelogenous leukemia (CML) (7). Neither of these cell lines showed evidence of myeloid differentiation in suspension culture. Recently, we identified a source of conditioned medium (CM) from one culture of whole human embryo cells (WHE-1 cells), which contained factor(s) capable of initiating and sustaining the exponential growth of myeloid leukocytes from patients with myelogenous leukemia. Successful results were achieved in 21 successive attempts with peripheral blood or bone marrow from 16 patients with AML or CML, while no growth was observed of blood or bone marrow leukocytes from 20 normal donors (48).

The purpose of the present report is 3-fold: first, to describe the properties of leukocyte cultures derived from the blood of the patient with AML whose cells produced a type-C virus after 4–10 weeks in culture (8); second, to provide available information about the WHE-1 cell CM; and third, to report the re-identification of apparently the same virus after 5 week's culture of leukocytes derived from a bone marrow specimen obtained when this patient was relapsing from chemotherapy-induced remission.

MATERIALS AND METHODS

Patient Information. The leukemic leukocytes used in this study came from a 61-year-old Caucasian female, designated HL-23, with AML. The initial peripheral blood specimen (8) was obtained at the time of diagnosis and prior to chemotherapy, when the white blood cell count was elevated to 125,000 cells per mm³ with approximately 70% undifferentiated leukemic myeloblasts. Subsequently, the patient was treated with cytosine arabinonucleoside, vincristine, and prednisone, and she experienced an extended remission. A second blood specimen and a bone marrow aspirate were obtained 14 months later when the patient was relapsing with a white blood cell count of 40,000/mm³ but with only approximately 10% blast cells in either specimen.

Preparation of Whole Human Embryo Cell Conditioned Medium. An ampule of frozen cells labeled WHE-M passage 2 (subsequently called WHE-1) was received from Dr. R. Ting (Biotech Labs., Rockville, Md.). Although records pertaining to the exact origin of these fibroblastic cells were not available, cyogenetically they were diploid human. For the preparation of CM, the WHE-1 cells were grown to confluence in T60 plastic flasks (Falcon Labs, Oxford, Calif.) in antibiotic-free McCoys 5A medium (GIBCO, Grand Island, N.Y.) containing 10% fetal calf serum (Reheis, Phoenix, Ariz.) The medium was changed and harvested 48 hr later, at which time potent leukocyte-stimulating activity was noted. Active CM was obtained through approximately 20 passage generations of the WHE-1 cells. Seeds of these cells were stored in a freezer which thawed, after which the CM potency was markedly decreased. Both the CM and WHE-1 cells were examined for mycoplasma by electron microscopy, by enrichment culture, and by biochemical analysis (9) with negative results. No type-C virus could be detected in either of these sources by electron microscopy, by attempts to infect numerous leukocyte and fibroblast cell cultures, or by assays for reverse transcriptase or p30 protein (assayed by C. Sherr and G. Todaro and by R. Gilden).

Leukemic Leukocyte Cultures. Cultures were maintained at 37° in a humidified, 5% CO₂ atmosphere. Leukocytes (1.25 × 10⁹) were seeded in T30 plastic flasks in 5 ml
RESULTS

Growth Kinetics in Liquid Suspension Culture. Strains I-1 and I-2 grew similarly (Fig. 1A). After a lag period of 3 days, the cells increased 25-fold by day 8. This corresponds to a cell doubling time of approximately 24 hr during the log phase of growth. Cell growth was dependent on the presence of WHE-1 CM (Fig. 1A) throughout the culture period. After a cell density of approximately 5 to 7 × 10^6 cells per ml was reached, further growth was not observed despite refeeding with additional CM. However, when such leukocytes were split into new flasks at 2.5 × 10^5 cells per ml, the same logarithmic growth pattern was repeatedly observed (Fig. 1B). Because of a limited supply of WHE-1 CM, none of the HL-23 leukocyte strains were maintained in culture for more than 4 months and detailed kinetic and cytological analyses have been limited to strains I-1 and I-2, although the other strains have shown the same general features.

Cytological Properties of the Cultured Leukocytes. The leukocytes from strains I-1, I-2, IV-1, and V-1 grew almost entirely in the liquid phase of the culture as single cells or small clumps (Fig. 2A). Leukocytes from strain II-1, initiated after a year's storage, grew more as single cells which had a tendency to adhere to the plastic flask. In growth pattern, these cultures resemble established lymphoblastoid cell lines of the "B" cell variety (1, 2), but they were distinguished by cytomorphology (see below), by a lack of Epstein-Barr virus antigens (11), and by a low response in a test for cell surface complement receptors (12).

Differential cell counts were performed on Wright-Giemsa stained preparations of the cells after various intervals in culture. As shown in Table 1 and illustrated in Fig. 2B–D, the following changes from the fresh blood sample were noted in the differential cell count of strain I-2 by 97 culture days: (1) a moderate increase in the percentage of apparently mature granulocytes, including neutrophils, eosinophils, and basophils; (2) some increase in the proportion of histiocytic-like mononuclear cells; and (3) a marked decrease in the percentage of lymphoid cells. As shown in Fig. 2C and D, myeloid cells with varying stages of differentiation were frequently noted in small clusters around a monocytic cell. In addition, an occasional erythrocyte precursor was noted during the first few weeks, and some erythrocytes persisted in the cultures.

![Figure 1](image1.png)

**FIG. 1.** (A) Growth of leukocytes in suspension culture from AML patient HL-23. Cells were seeded at 2.5 × 10^6 cells per ml, and cultured as described in Materials and Methods. 0, strain I-1 with conditioned medium; ●, strain I-2 with conditioned medium; △, either strain in the absence of conditioned medium. (B) Theoretical cumulative yield of HL-23 leukocytes in tissue culture. The plotted values are calculated from the increase in cell number in individual 5 ml flasks at each passage generation. They indicate the number of cells which would have been realized if all of the cells produced in one passage generation were seeded into fresh flasks at 2.5 × 10^5 cells per ml and successively propagated. This pattern continued without significant change through at least 11 passage generations for strain I-1 and I-2.

![Figure 2](image2.png)

**FIG. 2.** Cytological characteristics of fresh and cultured HL-23 leukocytes. (A) Characteristic appearance of growing HL-23 leukocytes in liquid suspension culture as noted in an inverted microscope (×240). (B) Smear preparation of the fresh peripheral blood specimen obtained from patient HL-23. Immature leukemic myeloid cells as well as a probable erythroid precursor cell are depicted (×720). (C) Small cluster of cultured HL-23 leukocytes obtained at the 97th culture day from strain I-2. The cells were mounted by dispersing the cultured leukocytes with a pipette and centrifuging at 500 rpm for 5 min in a Shandon-Elliott cytopsin centrifuge (model SCA-0030, Surrey, England). The "rosetting" of myeloid cells in various stages of differentiation around a large phagocytic monocytic cell (macrophage) was typical (×950). (D) Mature neutrophil and two unclassified mononuclear cells from the same preparation as in C (×1200). All illustrated stains are Wright-Giemsa.
Karyotype Analysis of the Cultured Leukocytes. Twenty to 50 metaphase figures were examined from all strains. Most of these from strains 1-1 and I-2 were normal female. However, a few metaphases from strains I-1 and I-2 and a majority of metaphases from strain II-1 showed either an abnormal condensation or a deletion of part of a G-group chromosome. A mixture of female karyotypes, some normal, some containing the G-group abnormality, was similarly observed in the fresh bone marrow obtained from the patient at diagnosis (J. Trujillo, personal communication). All metaphases examined from cultures derived from the second blood sample (strain IV-1) or from the bone marrow sample (strain V-1) were female diploid, as were metaphases examined from the fresh bone marrow at this time in the patient’s disease course (Trujillo, personal communication).

Description of WHE-1 Cell Conditioned Medium. Although the limited supply of WHE-1 CM (see Materials and Methods) has prevented a detailed analysis, it had the following notable properties: (1) stable for several months at -20° and several weeks at 4°; (2) not destroyed by repeated freezing and thawing; (3) retained activity at 37° for 24 hr and 56° for 30 min but is destroyed at 70° for 30 min; (4) nondialyzable for 72 hr in phosphate-buffered saline; (5) activity is not removed by ultracentrifugation at 150,000 × g for 1 hr; (6) required use with serum and an enriched growth medium, e.g., RPMI 1640, for a potent effect; (7) stimulated growth in liquid suspension culture of leukocytes from patients with myelogenous leukemia but not from the blood or marrow of normal donors; and (8) failed to stimulate growth of leukemic or normal myeloid cells in soft agar or methylcellulose.

Re-Identification of Type-C Virus. As previously reported (8) a typical type-C virus was identified in three substrains of cultured leukocytes from a blood sample obtained at diagnosis. The details of the passage history of these strains (I-1, I-2, and II-1) in relation to DNA polymerase assays which were performed on medium harvested from these strains are shown in Fig. 3. The asterisks indicate the points at which budding type-C virus was identified by electron microscopy and at which reverse transcriptase was characterized biochemically and immunologically (8, 13).

When virus was identified in cultures from the initial blood leukocyte cultures, a second blood specimen and a bone marrow aspirate were obtained. At this time the patient was in partial remission with approximately 10% leukemic myeloblasts in both blood and marrow. As indicated in Fig. 3, virus was not detected in the cultured blood specimen (strain IV-1) through 7 passages, at which time the specimen was frozen. However, typical budding type-C virus (Fig. 4 inset) as well as reverse transcriptase immunologically related to simian sarcoma and gibbon leukemia virus reverse transcriptase (13) were identified by the 5th passage of the bone-marrow-derived culture (strain V-1).

Electron Microscopic Analysis of the Virus-Producing Cell. The virions were produced in all strains by undifferentiated blast cells (Fig. 4). These cells were characterized by

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**Table 1. Differential cell counts of fresh and cultured leukocytes from patient HL-23**

<table>
<thead>
<tr>
<th>Days cultured</th>
<th>Blast cells</th>
<th>Immature myelocytes</th>
<th>Mature myelocytes*</th>
<th>Monocytes</th>
<th>Lymphocytes</th>
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<tr>
<td>0</td>
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<td>97</td>
<td>47</td>
<td>18</td>
<td>23</td>
<td>10</td>
<td>2</td>
</tr>
</tbody>
</table>

* Neutrophils, basophils, and eosinophils were observed.

**Fig. 3. Schematic history of leukocyte suspension cultures from the peripheral blood and bone marrow of patient HL-23 with acute myelogenous leukemia. Each arrow represents a passage generation of 7–10 days. The asterisks indicate the points at which type-C virus was identified by electron microscopy. The number to the right of the arrow indicates the DNA polymerase activity (pmol [H]TMP incorporated per ml of unconcentrated culture fluid per hr of reaction) in the presence of synthetic primer-template oligo(dT)-poly(A). The numbers in parentheses indicate the polymerase activity using oligo(dT)-poly(A) relative to that with oligo(dT)-poly(dA). When this ratio is greater than 1, it is suggestive that reverse transcriptase is present, although more definitive tests are required to prove this (23).**
relatively abundant cytoplasm containing many mitochondria but no organelles indicative of differentiation. The nuclei also appeared undifferentiated, frequently containing one or two nucleoli and little heterochromatin. No virus production was detected in more mature, granulated myeloid cells.

**DISCUSSION**

**Conditioned Medium-Dependent Growth of AML Cells.** *In vitro* studies suggest that normal granulopoiesis is regulated by chemical mediators, such as colony-stimulating activity or CSA (14-16, 5). In AML, this process is disturbed, and granulocyte precursors (myeloblasts) fail to differentiate normally. This is thought to be due to an intrinsic defect in leukemic blast cells but in some cases it could involve a deficit or abnormality of external stimuli for myeloid differentiation (5, 16-18). In this regard, the effect of CSA on either the proliferation or differentiation of leukemic myeloid cells is controversial (5, 16-22).

In this context, we emphasize the following points about the culture system described here. First, the magnitude and duration of the myeloid cell proliferation and differentiation in response to WHE-1 CM substantially exceeded that previously reported for CSA-dependent leukocyte growth in either semi-solid medium (5, 14-22) or liquid suspension culture (2-5). The established myeloblastic cell culture lines from an AML patient (6) and from a CML patient (7) did not require CM, did not differentiate in suspension culture, and demonstrated chromosomal aneuploidy, suggesting that these lines represent further progression of the neoplastic process. Second, the WHE-1 CM had apparent specificity for leukemic myeloid cells, since it promoted exponential growth in suspension culture of leukocytes from both AML and CML patients but had no stimulatory effect on peripheral blood or bone marrow cells from normal donors. Also, unlike CSA, it failed to stimulate growth of either normal or leukemic myeloid cells in semi-solid agar. There have been a few previous reports of CM from leukocyte cultures which selectively stimulated proliferation of AML cells (3, 5, 25). Such specificity might provide useful in *vitro* assays for distinguishing normal and transformed (leukemic) human myeloid leukocytes. Third, the WHE-1 cell CM was unusual in its source. Most human CSAs have been derived from blood leukocytes (3, 5, 21-27) or spleen cells (17). To our knowledge, short-term cultures of embryonic kidney cells are the only reported source of CSA from passaged human tissue culture cells (20). We obtained CM produced by these embryonic kidney cells (Flow Labs, Rockville, Md.) and found that it had no activity in our culture system. In addition, because of the loss of CM factor production by the WHE-1 cell strain, we have tested 18 other early-passage WHE cell cultures without detecting potent granulocyte-stimulating activity (F. Ruscetti, unpublished data). Finally, the specificity of the WHE-1 CM suggested that the cultured granulocytes from patient HL-23 which appear morphologically mature (Fig. 2C and D) were derived from leukemic blast cells. If so, it would confirm previous reports that some leukemic myeloblasts may mature (17, 19, 21) if the proposed block in differentiation is removed. The kinetic and karyological studies in this case support this possibility, but in the absence of detailed kinetic analyses of specifically identified leukemic cells, we cannot definitely exclude the alternative possibility that the mature granulocytes were derived from normal precursors in the leukemic blood specimen.

**Type-C Virus Production by the Cultured AML Cells.** There are three possible explanations for the production of type-C virus by the cultured HL-23 leukocytes: (1) an increase during tissue culture of the amount of complete type-C virus produced at an undetectable level by the fresh leukemic cells; (2) assembly of subviral components, some of which have previously been detected in fresh AML blood cells (23-31), into mature extracellular type-C virions; or (3) infection of the HL-23 leukocytes by a laboratory-grown viral contaminant. The present identification of apparently the same virus (13) in a second specimen from patient HL-23 provides further evidence that the third possibility of accidental virus contamination is remote (8). These results, however, do not discriminate between the first and second possibilities. The pattern of increasing virus production noted in the various substrains of HL-23 cells with sequential tissue culture passaging (see Fig. 3) is consistent either with the activation of incompletely expressed virus analogous to the induction or spontaneous release of virus from nonproducer animal cells in tissue culture (32, 33) or with the augmented production of a latent, preformed type-C virus (34). Our failure to observe type-C virus particles in whole fresh leukocytes or in reverse-transcriptase-containing fresh blood cell homogenates of patient HL-23 could be due to inadequate sensitivity of the electron microscopic technique. In this regard, particles with the morphological features of type-C virus have been observed in fresh human leukemic leukocytes (35). The type-C particles continuously released by the cultured HL-23 leukocytes should, however, be distinguished from "virus-like" particles which have been detected in homogenates of fresh AML cells (23-30) or which have been transiently released into tissue culture fluids after short-term culture of leukemic leukocytes (36, 37). Although such particles contain reverse transcriptase (24-28) and viral-related nucleic acids (23-30), they have not been reported to have some important morphological characteristics of type-C viruses, including cell membrane budding, or any biological property of known RNA tumor viruses, including infectivity or virus replication.

As reported elsewhere by Teich, et. al. (13), virus produced by the HL-23 cultured leukocytes was infectious for several homologous and heterologous fibroblastic cell culture lines. These cell lines, which produce virus at substantially higher titer than the primary leukocyte cultures, are being grown to prepare sufficient quantities of viral molecular components for comparison of the isolates of type-C virus.
from patient HL-23 to known primate type-C viruses and to other human candidate RNA tumor viruses (see below). Virus produced by the primary HL-23 leukocyte culture strains has been shown to contain reverse transcriptase (8, 13) and p30 protein antigenically related to these components from two tumorigenic type-C viruses isolated from sub-human primate tumors (38, 39), the woolly monkey (simian) sarcoma virus (SISV) and gibbon ape leukemia virus (GALV). The same immunological results for reverse transcriptase (40, 41, 23) and p30 protein (31) had previously been found with fresh blood leukocytes from some patients with AML, including this patient (42).

Since the initiation of the present studies, we have learned of other isolates of type-C viruses from human cells in three independent laboratories. These include two isolates from a child with acute lymphosarcoma leukemia (43), an isolate from co-cultures of rat (XC) cells with cells derived from a lung tumor of an adult with concurrent chronic lymphatic leukemia (44), and several isolates from multiply passaged cultures of whole human embryo cells derived from putatively normal first trimester abortuses (45). Initial results indicate that all of these isolates are related to GALV and, particularly, to SISV (43–45). A thorough comparative analysis of these virus isolates and their tissues of origin may advance our understanding of the possible role of RNA tumor viruses in human leukemia.

We thank Dr. R. Ting and Ms. C. Sun (Biotech Labs, Rockville, Md.), Dr. J. Whang-Peng (National Cancer Institute (NCI), Bethesda, Md.) and Dr. J. Trujillo (M. D. Anderson Hospital, Houston, Texas) for karyotype analyses. Also, we thank Dr. B. Hamburger (NCI) for performing Epstein–Barr nuclear antigen tests (11) for that virus, Dr. E. Shevach (NIAID) for performing the EAC test (12) for cell surface complement receptors, Drs. W. Blattner (NCI) and F. Ruscetti (Litton) for helpful discussions, Dr. J. Anderson (Salina, Kansas) for assistance in the procurement of specimens and clinical information, and Mr. A. Engle for technical assistance. This work was supported by the Division of Cancer Treatment and by the Virus Cancer Program of the NCI.