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Isolation of a type C RNA virus from an established human histiocytic lymphoma cell line

(RNA tumor virus/human malignant lymphoma/reverse transcriptase/mixed culture cytopathogenicity)

HENRY S. KAPLAN, ROBERT S. GOODENOW, ALAN L. EPSTEIN, SUZANNE GARTNER, ALAIN DECLÈVE, AND PETER N. ROSENTHAL

Cancer Biology Research Laboratory, Department of Radiology, Stanford University Medical Center, Stanford, California 94305

Contributed by Henry S. Kaplan, April 8, 1977

ABSTRACT **A type C RNA virus has been detected in the culture fluids of the SU-DHL-1 human histiocytic lymphoma cell line previously established in this laboratory. In electron micrographs, the virus closely resembled other typical mammalian type C RNA tumor viruses in size and morphology. Viral RNA-dependent DNA polymerase activity has been demonstrated in particles (densities of 1.15 and 1.22 g/ml) in the microsomal cytoplasmic fraction and in pellets of culture fluids. The enzyme is partially inhibited by antibodies to the RNA-dependent DNA polymerases of simian sarcoma virus and RD-114 virus but not by antibody to the polymerase of murine leukemia virus, suggesting some degree of relatedness to type C viruses of subhuman primate origin. Typical syncytial microplaques were induced when SU-DHL-1 cells were cocultivated with rat XC cells. Although no focus formation was noted in similarly cocultivated mouse UC1-B cell cultures, the numbers of foci induced in rat embryo fibroblasts by murine sarcoma virus were significantly increased by coinfection with the virus from SU-DHL-1 cell culture fluids. No other evidence of infectivity, inducibility, or capacity for helper rescue of defective murine sarcoma virus genomes has been detected to date in cocultivation studies with a spectrum of fibroblastic and other nonlymphoid indicator cell lines of human and other species of origin.**

Type C RNA viruses have been identified as causative agents of leukemias, lymphomas, and sarcomas in avian, murine, feline, cavian, bovine, and subhuman primate species (cf. ref. 1). These observations have stimulated interest in the possibility that such viruses may also be etiologic agents in the corresponding human malignant diseases. Type C viruses have been detected in culture fluids after short-term culture of hemopoietic cells of patients with leukemia (2–4) and after cocultivation of rat XC cells with lung carcinoma cells from a patient with concurrent chronic lymphocytic leukemia (5). The spontaneous release of such viruses from two diploid human embryonic cell strains after a few months in culture has also recently been reported (6). The viral isolates obtained in these studies contained RNA genomes with extensive sequence homologies to the genomes of both simian sarcoma virus and an endogenous virus of baboons (7, 8). In addition, some of these isolates have been shown to contain structural proteins and RNA-dependent DNA polymerase (RDDP) immunologically related to the corresponding proteins of the simian sarcoma virus and the baboon virus (7, 8).

Components of incomplete type C viruses have also been detected in human neoplasms. Leukemic cells have been reported to contain intracytoplasmic particles with a density characteristic of type C viruses and an enzymatic activity indistinguishable from that of RDDP (2, 9, 10). Viral polymerase has been purified from the spleens of patients with chronic lymphocytic leukemia and chronic myelogenous leukemia (11), and the simultaneous detection assay for both 70S RNA and RDDP (12) has reportedly been positive in spleen tissue from patients with various types of leukemia and Hodgkin's disease (13). A serious obstacle to further characterization of the type C viral components in human neoplastic cells has been the need to rely upon the inconsistent quality and sporadic availability of primary human tissue specimens. The detection of a new herpes-type agent, the Epstein–Barr virus, was made possible by the establishment of African Burkitt lymphoma cells in continuous culture (14, 15). Accordingly, studies of the biology and virology of other human malignant lymphomas in this laboratory began with the cultivation *in vitro* of three diffuse histiocytic lymphomas (16). The isolation and initial characterization of a type C RNA virus from the culture fluids of one of these permanently established human malignant lymphoma cell lines and the detection of particle-associated RDDP activity and 70S RNA in microsomal fractions prepared from the tumor cells are the subjects of this report.

Abbreviations: RDDP, RNA-dependent DNA polymerase; M-MuLV, Moloney murine leukemia virus; GaLV, gibbon ape leukemia virus; SSV-1, simian sarcoma virus; FeLV, feline leukemia virus; BaEV, baboon endogenous virus; MSV, murine sarcoma virus; LREF, Lewis rat embryo fibroblasts; FFU, focus-forming units.

MATERIALS AND METHODS

Cells. The SU-DHL-1 cell line was established in this laboratory from the malignant pleural effusion of a 10-year-old Caucasian boy with diffuse histiocytic lymphoma (16). The cultured tumor cells were readily distinguishable from lymphoblastoid cells by their aneuploid karyotypes, heterotransplantability, histochemical features, lack of thymus-independent lymphocyte (B cell) surface markers, and absence of the Epstein–Barr virus nuclear antigen (16). The cell line has been grown in continuous suspension culture for over 2 years and has remained free of mycoplasma contamination.

Viruses. The Moloney murine leukemia virus (M-MuLV) was propagated in rat cell line 78A-1 and purified on sucrose density gradients (17). Gibbon ape leukemia virus (GaLV) and simian sarcoma virus (SSV-1) grown on either NC37 or A204 cells, feline leukemia virus (FeLV) grown on FL-64 cells, RD-114 endogenous feline virus propagated on RD cells, and baboon endogenous virus (BaEV) grown on BKCT cells were doubly banded in sucrose density gradients (supplied by Pfizer, Inc., Maywood, NJ, through the Resources and Logistics Program of the National Cancer Institute). The NIH pseudotype of murine sarcoma virus (NIH-MSV) was obtained from J. Levy, University of California, San Francisco, CA.

XC Cell Assay. The reverse XC cell assay procedure described by Niwa *et al.* (18) was modified by the addition of graded numbers of SU-DHL-1 cells on day 1, soon after the XC cells had become attached. In some experiments, the SU-DHL-1 cells were irradiated with a dose of 10,000 rads from a 200 Ci ^{137}Cs irradiator. Medium was changed at day 4, at which time

most of the SU-DHL-1 cells were removed. The XC cells were harvested (P_0) at day 5 or 6 or subcultured and harvested (P_1) at day 9 or 10, when they again became confluent. The cells were fixed for 10 min in methanol, stained for 20–30 min with Giemsa, and examined for syncytia at ×50 magnification.

Cocultivation, Induction, and Infectivity. Six cocultivation experiments (CC-I–CC-VI) were performed with SU-DHL-1 cells and KHOS, a nonproducer human osteogenic sarcoma cell line infected with the Kirsten murine sarcoma virus (19). The cells were grown in flask cultures at SU-DHL-1/KHOS cell ratios ranging from 5:1 to 50:1. SU-DHL-1 cells were also cocultivated (CC-VII) for 48 hr with the following cell lines: KHOS, kindly provided by J. S. Rhim, Microbiological Associates, Bethesda, MD; 1016B (human teratoma) kindly provided by B. McCaw, University of Oregon Medical Center, Portland, OR; A204 (human rhabdomyosarcoma), 8155 (fetal canine thymus), and DBS-1 (fetal rhesus lung) kindly supplied by J. E. Verna, Meloy Laboratories, Springfield, VA; AU302 (human fibrosarcoma) and A0676 (human osteogenic sarcoma) kindly provided by W. Nelson-Rees, Naval Biomedical Research Laboratory, Oakland, CA; and 64J1 (transformed mink lung) kindly provided by C. Sherr and G. Todaro, National Cancer Institute. Dexamethasone (10 μg/ml) was added to some cultures for 24 hr in CC-III and CC-V, and 2-deoxyglucose (5 mg/ml) and 5-iododeoxyuridine (IdUrd, 20 μg/ml) were added to other cultures in CC-III. At 48 hr after the start of cocultivation in CC-VII, IdUrd at the same concentration was added for 24 hr. All cultures were serially subpassaged at confluence and periodically monitored for the appearance of reverse transcriptase activity in the supernatant fluids. In addition, infectious center assays were performed in CC-IV by plating the cocultivated cells with mink lung cells, Lewis rat embryo fibroblasts (LREF), and a normal human skin fibroblast line, SU-NSF-1. These cells were then processed for the detection of cytoplasmic viral antigens by indirect immunofluorescence, with a rat anti-MuLV serum, as previously described (20). One cocultivation experiment was performed as previously described (21) with UC1-B, a Balb/3T3 fibroblastic line which forms foci when superinfected with other mammalian type C viruses (22(/ The effect of coinfection with NIH-MSV and the virus from SU-DHL-1 cell culture fluids was tested on several cell lines, of which only LREF yielded foci. Cultures passaged twice after infection with concentrated SU-DHL-1 culture fluids and virus-free control cultures were reseeded at 2×10^5 cells per dish, incubated for 24 hr, and then infected with an estimated 10 focus-forming units (FFU) of NIH-MSV (in 0.4 ml) for 2 hr. Fresh medium was added and all cultures were subpassaged at 4 days. Focus counts were made at 8 days with an inverted microscope.

Isolation of the Microsomal Pellet. Cultured cells were washed three times with cold phosphate-buffered saline and resuspended in 0.15 M NaCl/0.01 M Tris·HCl, pH 7.8/10 mM EDTA containing 0.5 mM phenylmethylsulfonylfluoride. The cells were Dounce-homogenized eight times, and the disruption was monitored by light microscopy. Microsomal pellets were then prepared by the procedure of Gulati *et al.* (23).

Polymerase Assays. Assays for viral reverse transcriptase in cell culture fluids and in sucrose gradients were performed as described by Lieber *et al.* (24), modified by incubation at pH 8.1 and 30° with 0.25 A_{260} unit of $(rA)_n$ and 0.025 A_{260} unit of $(dT)_{12-18}$ per ml.

DEAE-Cellulose Chromatography. Microsomal pellets were chromatographed on a 10-ml column of DEAE-cellulose (Whatman DE-52) as described by Witkin *et al.* (11). Viral polymerases were chromatographed similarly and the active fractions were stored in 50% (vol/vol) glycerol at −20°.

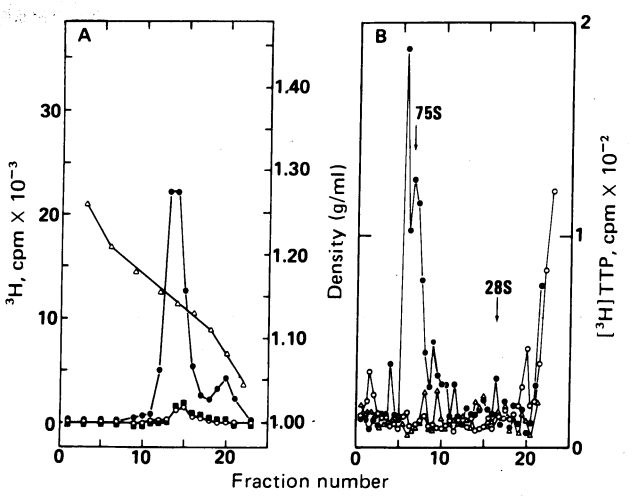

FIG. 1. RNA-dependent DNA polymerase (RDDP) activity of the microsomal pellet from SU-DHL-1 cells. (*A*) The microsomal pellet from 10^9 SU-DHL-1 cells was prepared as described in *Materials and Methods* and fractionated by sedimentation to equilibrium in a 10–70% sucrose gradient (17). Twenty-three fractions were collected from the bottom of the tube and 25-μl aliquots were assayed for RDDP activity with $(rA)_n \cdot (dT)_{12-18}$ (●), as described in *Materials and Methods;* and with $(rC)_n \cdot (dG)_{12-18}$ (○), assayed similarly except at 37° and with [^{3}H]dGTP replacing [^{3}H]dTTP. Endogenous activity (■) was assayed at 37° in 200 μl of 40 mM Tris·HCl, pH 8.1/50 mM KCl/5 mM dithiothreitol/1 mM $MnCl_2$/0.1 mM each of dATP, dCTP, dGTP/0.1% Triton X-100/$(dT)_{12-18}$, 10 μg/ml/[^{3}H]TTP, 25 μCi/ml (20 Ci/mmol). Density (△) was determined by refractometry.

(*B*) Sedimentation velocity analysis of the endogenous product synthesized by the microsomal pellet from SU-DHL-1 cells. Aliquots (100 μl) of a microsomal pellet were adjusted to 0.1% Triton X-100 and added to a 200-μl assay mixture. The complete endogenous reaction mixture was modified by omitting $(dT)_{12-18}$ and Triton X-100 and adding actinomycin D (100 μg/ml) and $MnCl_2$ to 5 mM. After 15 min at 37° the reaction was terminated by adding 1% sodium dodecyl sulfate, 0.5 M NaCl, and Pronase (0.8 mg/ml) and incubating at 37° for 45 min. Bacteriophage PM-2 [^{14}C]DNA-I was added to each sample as an internal 28S sedimentation marker (32). Samples were sedimented on separate sucrose gradients and assayed for radioactivity (33). ●, complete reaction mixture; △, complete reaction mixture minus dGTP; ○, complete reaction mixture subsequently treated with 20 μg of ribonuclease for 15 min at 25°. Arrows denote the mean position of the 28S [^{14}C]DNA marker and the calculated position corresponding to 75 S.

Antibodies to RNA-Dependent DNA Polymerases. Antisera against purified viral RDDP were generously provided by the following investigators: anti-GaLV, C. Sherr and G. Todaro, National Cancer Institute; anti-SSV-1, S. Tronick and S. Aaronson, National Cancer Institute; anti-M-MuLV and anti-RD114, C. Long, Flow Laboratories, Rockville, MD.

Electron Microscopy. Culture fluids from 1.5 liters of exponentially growing SU-DHL-1 cells were clarified by centrifugation at 4000 × *g* for 20 min at 4° and concentrated by centrifugation at 50,000 × *g* for 150 min at 4° in a Beckman type 19 rotor. The pellet was resuspended in 2 ml of 0.1 M NaCl/0.01 M Tris·HCl, pH 7.5/1 mM EDTA and isopycnically banded in a linear sucrose gradient containing the same buffer (16). Electron micrographs of the negatively stained, banded material were kindly prepared by J. Griffith, Department of Biochemistry, Stanford University School of Medicine.

Reagents. Ribonuclease and Pronase were purchased from Calbiochem. Synthetic polynucleotides were obtained from P. L. Biochemical and Miles Laboratories. Sterox SL was kindly provided by the Monsanto Co. Radioisotopes and Omnifluor were purchased from New England Nuclear Corp.

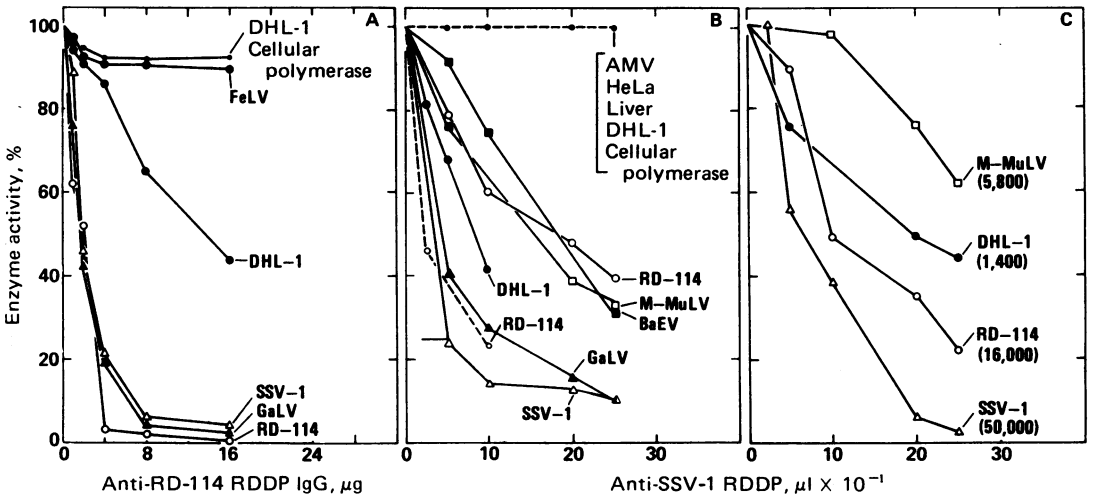

FIG. 2. Characterization of cellular and viral RNA-dependent DNA polymerases from SU-DHL-1 cells with inhibitory antisera. The reaction conditions using $(rA)_n \cdot (dT)_{12-18}$ primer-template and the partial purification of enzyme on DEAE-cellulose as well as the sources for viruses and antisera are all described in *Materials and Methods*. For inhibition studies, 50 μl of RDDP partially purified on DE-52 or 50 μl of sucrose-banded virions in 0.1% Triton X-100 was mixed with 20 μl of graded concentrations of immune serum or immune IgG and incubated for 10 min at 0°. Then, 100 μl of complete reaction mixture was added, and incorporation was measured after incubation for 60 min at 30°. The percentage enzyme activity is calculated relative to the extent of synthesis in a parallel tube with the respective polymerase and nonimmune serum or nonimmune IgG. (*A*) Inhibition of SU-DHL-1 RDDP partially purified on DE-52 by the IgG fraction of an antiserum prepared against RD-114 RDDP. The SU-DHL-1 enzymes were prepared from the microsomal pellet. The SU-DHL-1 cellular DNA polymerase eluted from DE-52 after the RDDP activity and preferentially utilized $(dA)_n \cdot (dT)_{12-18}$. (*B*) Inhibition of RDDP partially purified on DE-52 by an antiserum to RDDP from SSV-1. The enzymes were prepared as described above. Purified avian myeloblastosis virus (AMV) RDDP, HeLa cell DNA polymerase γ, and human liver DNA polymerase β were the gifts of J. Beard, A. Weissbach, and D. Korn, respectively. Purified RD-114 virions (O- - -O) were disrupted as described below. (*C*) Inhibition of the RDDP from extracellular type C particles in SU-DHL-1 cell culture fluids by antiserum to SSV-1 RDDP. SU-DHL-1 particles were concentrated from culture fluids as described in *Materials and Methods*, disrupted in 0.1% Triton X-100, and assayed as described above. Purified murine, feline, and simian viruses were similarly disrupted and assayed. Numbers in parentheses indicate ^3H incorporation in control tubes with nonimmune serum.

RESULTS

Detection of RDDP Activity Associated with a Particle of Oncornavirus Density. Microsomal pellets prepared from SU-DHL-1 cells were banded to equilibrium in sucrose density gradients. the gradient fractions were assayed for RDDP with three different primer-templates (Fig. 1*A*). Most of the RDDP activity banded in the 1.13–1.15 g/ml density region of the gradient. With $(dT)_{12-18}$ and only one nucleoside triphosphate, [^3H]dTTP, no synthetic activity was detected; this excludes terminal deoxynucleotidyl transferase as the source of the observed enzymatic activity (25). An additional peak of enzymatic activity in the 1.22–1.25 g/ml density region was observed in only one instance. Slight displacement of the $(rA)_n \cdot (dT)_{12-18}$ peak relative to the $(rC)_n \cdot (dG)_{12-18}$ and endogenous activities was noted in one experiment.

SU-DHL-1 cell microsomal pellets were also assayed by the simultaneous detection procedure (12) in the presence and absence of actinomycin D, and a ribonuclease-sensitive tritiated product sedimenting at 70–80 S was found. The synthesis of this material was dependent upon the presence of all four deoxynucleoside triphosphates (Fig. 1*B*).

Detection of RDDP Activity in Supernatant Culture Fluids. Periodic assays of the culture fluids from both cocultivated and SU-DHL-1 control cultures revealed low but significant and comparable levels of RDDP activity. An ampule of SU-DHL-1 cells frozen at day 40 after initiation of the cells in culture was thawed and cultured, and similar levels of enzymatic activity were observed in the supernatant fluids of these early subpassage cells. RDDP activity has also been detected at comparable levels in the supernatant fluids of five subclones of SU-DHL-1 cells.

Partial Characterization of the RNA-Dependent DNA Polymerase Activity. The RNA-dependent DNA polymerase from the microsomal pellet of SU-DHL-1 cells, as well as the enzyme obtained from the concentrated culture fluids of these cells, was further characterized by examining the capacity of antisera, prepared against purified RDDP from several mammalian type C viruses, to inhibit enzymatic activity. The DEAE-cellulose-purified enzyme from the lymphoma cell microsomal pellet was only partially inhibited by antibody to the polymerases of RD-114 and SSV-1, at antiserum concentrations that inhibited more than 90% of the homologous enzyme activity (Fig. 2 *A* and *B*). Similar partial inhibition of the RDDP activity obtained from concentrated culture fluids of these cells was observed with antisera to SSV-1 RDDP (Fig. 2*C*) and RD-114 RDDP (data not shown). In contrast, antiserum to the RDDP of M-MuLV had no inhibitory activity against either source of the SU-DHL-1 RDDP, even at IgG concentrations that were >90% inhibitory for the homologous murine viral enzyme. Comparable patterns of inhibition were obtained with SU-DHL-1 RDDP prior to partial purification. No inhibition of cellular DNA polymerases α, β, or γ was observed with antiserum to SSV-1 RDDP (Fig. 2*B*). These data demonstrate that the lymphoma viral polymerase carries antigenic determinants common to the enzymes of both RD-114 and the subhuman primate viruses.

The RNA-dependent DNA polymerase was partially purified from the microsomal pellet of SU-DHL-1 cells by chromatography on DEAE-cellulose as described in *Materials and Methods*. The major peak of the activity stimulated by $(rA)_n \cdot (dT)_{12-18}$ eluted at 0.2 M KCl, ahead of the $(dA)_n \cdot (dT)_{12-18}$ activity attributed to the cellular polymerases. This activity eluting at 0.2 M KCl had an absolute requirement for a divalent cation and was more active with Mn^{2+} than with Mg^{2+}. With either cation, the enzyme was more active with $(rA)_n \cdot (dT)_{12-18}$ than with $(dA)_n \cdot (dT)_{12-18}$. No activity was

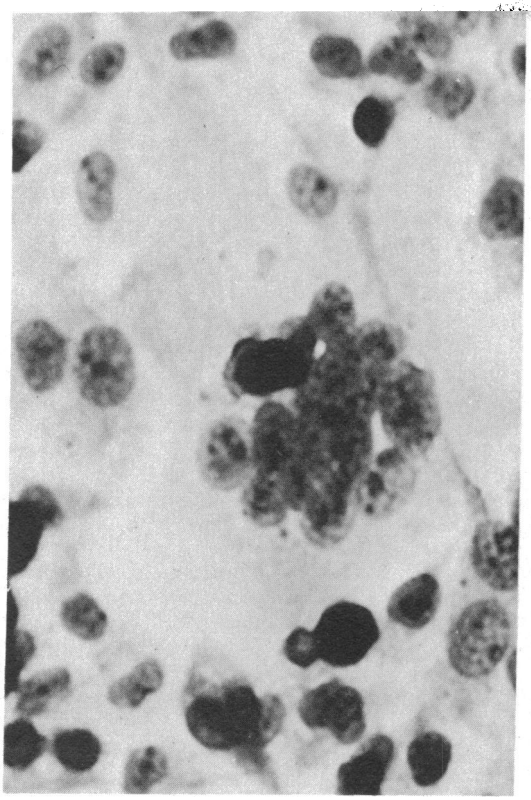

FIG. 3. Typical syncytium induced in rat XC cells by cocultivation with SU-DHL-1 cells.

detected with either $(A)_n$ or $(dT)_{12-18}$ alone. All of these primer-template preferences are consistent with the known properties of RNA-dependent DNA polymerases and exclude terminal deoxynucleotidyl transferase as the active enzyme in the microsomal pellet of SU-DHL-1 cells (25, 26).

Cocultivation, Chemical Induction, and Infectivity Studies. Treatment of SU-DHL-1 cell cultures with IdUrd or deoxyglucose failed to induce significant increases of RDDP activity in the culture fluids at 1, 2, 5, and 10 days after removal of the inducing agent. In one of six experiments, cocultivation of SU-DHL-1 cells with KHOS cells yielded a transient increase in RDDP activity in the supernatant culture fluids at day 13, with a return to base-line levels by day 19. Increased levels of RDDP activity have not been observed to date (>125 days) in the culture fluids of any of the indicator cell lines used in CC-VII.

In several replicate experiments, cocultivation of SU-DHL-1 cells with rat XC cells consistently induced syncytium formation (Fig. 3). The number of syncytia induced increased nonlinearly with the numbers of SU-DHL-1 cells plated on the XC cell monolayers and was unaffected by irradiation or by subpassage of the XC cells (Table 1). Focus formation was not observed in

Table 1. Reverse XC cell assay of SU-DHL-1 cell virus

No. of SU-DHL-1 cells plated	Mean no. of syncytia/plate*
10^4	22
10^5	40
10^6	243

* Mean of four experiments, two with the P_0 XC passage and two with the P_1 XC passage. The same four experiments with x-irradiated (10,000 rads) SU-DHL-1 cells gave similar results.

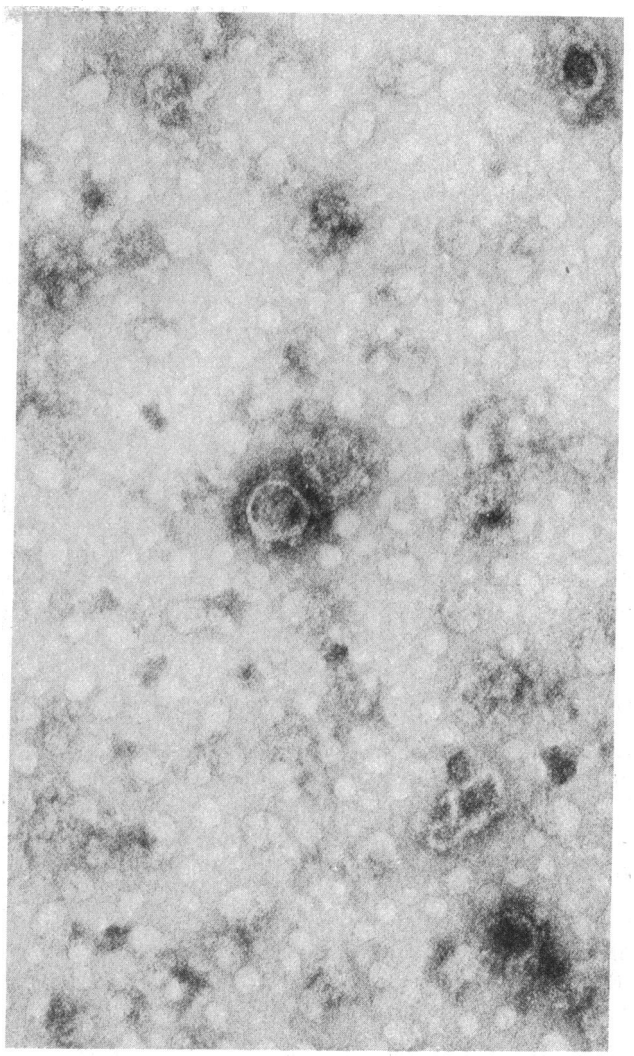

FIG. 4. Typical particles observed in pelleted SU-DHL-1 cell culture fluids. Their size and morphology are quite similar to those of other mammalian type C RNA viruses. This electron micrograph was kindly prepared by J. Griffith, Department of Biochemistry, Stanford University.

UC1-B cells after cocultivation with SU-DHL-1 cells. However, focus formation by NIH-MSV on LREF was significantly increased when the cells were preinfected with virus from SU-DHL-1 culture fluids (mean ± SD, 22.8 ± 6.3 FFU versus 6.0 ± 2.8 FFU).

Ultrastructure of the Supernatant Culture Fluid Particles. Particles isolated from SU-DHL-1 cell culture fluids and isopycnically banded were examined by electron microscopy after negative staining. Irregularly shaped spherical particles with a diameter of about 100 μm were observed (Fig. 4). They were composed of a moderately dense central nucleoid surrounded by an envelope, the inner layer of which was electron-dense. The outer envelope could be removed by treatment with a nonionic detergent, Sterox SL. The size, morphology, and detergent-sensitivity of these particles are very similar to those of type C RNA viruses isolated from other mammalian species (27).

DISCUSSION

A virus has been isolated from the culture fluids of a permanently established human malignant lymphoma cell line. The virus has an equilibrium density of approximately 1.15 g/ml

in sucrose gradients and has been shown to contain RDDP activity and RNA. In negatively stained electron micrographs, its size and morphology closely resemble those of other mammalian type C viruses. The virus exhibits the capacity to induce syncytia in rat XC cells, a biological attribute characteristic of murine (28), feline (29), and subhuman primate (30, 31) type C RNA viruses. Studies on the inhibition of its RDDP activity by antibodies to simian, endogenous feline, and murine viral polymerases suggest some degree of relatedness to type C viruses of subhuman primate origin. However, to date the virus has failed to propagate, in the presence or absence of chemical inducing agents, on a spectrum of fibroblastic and other nonlymphoid indicator cells lines or to rescue defective murine sarcoma viruses from cells bearing sarcoma virus genomes.

To our knowledge, this is the first reported instance of the spontaneous and sustained release of a type C RNA virus by a permanently established human neoplastic cell line. The L104 cell line described by Gabelman *et al.* (5), which releases a rat-tropic type C virus, was derived by cocultivation of rat XC cells with lung carcinoma cells from a patient with concurrent chronic lymphocytic leukemia, and it exhibits a karyotype indicative of hybrid, but primarily rat, derivation. The SU-DHL-1 cell line was established directly from a malignant pleural effusion from a patient with diffuse histiocytic lymphoma, without contact with cells of other than autologous origin (16). The virus has been detected in SU-DHL-1 cells frozen and stored after only 25 to 40 days in culture and in all subclones of this cell line, as well as in SU-DHL-1 cells maintained in continuous culture for more than 2 years. The availability of a permanently established human neoplastic cell line as the source of this type C RNA virus should greatly facilitate studies of its biological significance. Evidence has recently been obtained that the virus can induce proliferation of normal human monocytes and histiocytes *in vitro* (H. S. Kaplan, unpublished data).

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