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Characterization of Extracellular Particles Released from Continuous Cell Cultures Derived from Human Leukemia[1] (39459)

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Several studies reported oncornavirus-like particles in media taken from cultured cells derived from patients with malignant hemopoietic and lymphoid disorders (1-4). In contrast, particles with properties unlike oncornaviruses were detected in culture medium of cells derived from patients with Hodgkin's Disease (5, 6), adenocarcinoma (7), and the plasma of patients with chronic lymphocytic leukemia (8).

Barker *et al.* reported that continuous cell lines (Z-597, JIII) derived from human leukemias contained cytoplasmic particles with a density of 1.18 g/cm^3 in sucrose and a DNA polymerase, inhibitable by RNase (9). These contained RNA, DNA, and a covalently linked RNA-DNA complex (10).

The present report describes the detection and characterization of extracellular particles released into the medium of cultured JIII cells, which are distinctly unlike oncornavirions.

Materials and methods. Cell lines. The JIII cell line was derived from peripheral blood of a patient with monocytic leukemia and obtained from American Type Culture Collection Cell Repository, Rockville, Md. The Z-597 cell line was established by Dr. William Murphy, University of Michigan, Ann Arbor from peripheral blood of a patient with granulocytic leukemia. Cells were grown on a glass substrate at 37° with a mixture of 1× Eagle's minimal essential medium (11) and Hank's balanced salt solution (12) which contained 15% fetal calf serum (Flow Laboratories, Rockville, Md.) and antibiotics. Cells were passaged at 5-7 day intervals. Cell lines were free of Mycoplasma as shown by failure to culture aerobically and anaerobically the organisms from partially disrupted cells and culture medium, and by electron microscopic examination of cell cultures.

Processing of particles from tissue culture fluid. Tissue culture fluid was removed from cell monolayers and centrifuged twice at 1000*g* for 30 min at 4°. The supernatant was centrifuged at 55,000*g* in a Beckman Type-30 rotor for 1 hr at 4°. Pellets were resuspended in 0.5 ml of 0.1 *M* Tris-HCl (pH 8.3)-0.1 *M* EGTA and centrifuged to equilibrium in a 12-60% sucrose gradient for 18 hr at 90,000*g*. An aliquot from each fraction collected from the tube was assayed for either DNA polymerase activity or acid-insoluble radioactivity.

Biochemical procedures. Cells grown to a confluent monolayer were labeled with radioactive isotopes by changing the medium to 1× Eagle's with 2% fetal serum and adding either 0.2 μCi/ml of [*methyl*-^{3}H]thymidine (sp act 25 Ci/mmole) or [5-^{3}H]uridine (sp act 21 Ci/mmole) (Nuclear Dynamics). After 24 hr the medium was harvested and processed for particles.

DNA polymerase assays were done as described previously (9). Acid-insoluble radioactivity measurements were performed as described by Barker *et al.* (10).

Electron microscopy. Preparations were placed on Formvar carbon-coated copper grids, stained with 2% phosphotungstic acid, and examined with an AEI Corinth 275 electron microscope.

Immunological procedures. Particles processed from the culture medium were mixed with an equal volume of complete Freund's adjuvant and injected into the footpads of rabbits. Booster injections with a equal volume of Freund's adjuvant were given subcutaneously.

Globulins from the preimmune and immune rabbit serum were conjugated with fluorescein isothiocyanate (Baltimore Biological Laboratory, Cockeysville, Md.) by

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the method of Riggs *et al.* (13). All conjugates were absorbed with a solid matrix of cross linked bovine serum albumin in order to remove antibodies possibly elicited against the albumin fraction of fetal calf serum.

Cells to be examined for extracellular antigens were grown in Leighton tubes with coverslips, fixed in acetone for 10 min, and then tested with either the direct or indirect fluorescent antibody method. Unfixed cells were also tested for surface antigens using the direct fluorescent antibody method (equal volumes of cells and conjugate were incubated).

Result. Release of particles from JIII cells with RNA, DNA, and DNA polymerase. A pellet, sedimented by differential centrifugation from medium of JIII cell cultures, was resuspended and centrifuged to equilibrium in a sucrose gradient. Fractions of the gradient were assayed for DNA polymerase activity with endogenous nucleic acid templates and primers. A peak of enzyme activity was detected in the 1.18–1.22 g/cm^3 region of the gradient (Fig. 1). Prior to collection of the fractions, an optically visible band was observed in the gradient corresponding in position to the polymerase activity.

In another series of experiments, JIII cell cultures were labeled with either [^{3}H]thymidine or [^{3}H]uridine. The culture fluids were processed and centrifuged as described above, whereupon peaks of [^{3}H]thymidine or [^{3}H]uridine radioactivity were found in the 1.18–1.22 density region of the gradient. Thus, the RNA, DNA, and polymerase were coincident with the optically visible band of the gradient (Fig. 1).

Medium from Z-597 cell cultures also yielded particles similar to JIII cultures in density, DNA polymerase and [^{3}H]uridine incorporation. Since such particles could not be recovered from medium of normal skin fibroblast cultures or culture medium incubated without cells, it is concluded that the particles in the media of JIII and Z-597 cultures were released from the cells in those cultures.

Properties of the DNA polymerase activity. The DNA polymerase associated with JIII particles was assayed with varying concentrations (0.005–0.200%) of Nonidet, a nonionic detergent. The maximally effective concentration 0.1% increased the enzyme activity over sixfold (Table I). This stimulatory effect suggests that access of the enzyme to components of the assay mixture is limited by lipid.

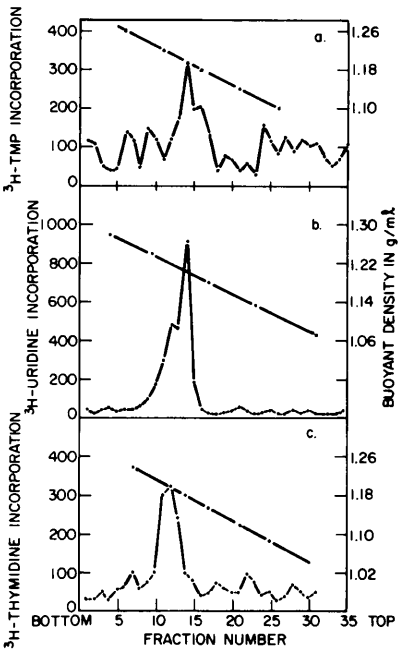

FIG. 1. Buoyant density of JIII particles determined by isopycnic gradient centrifugation. Cell cultures were either (a) unlabeled, (b) labeled with 0.2 μCi of [^{3}H]uridine or (c) labeled with 0.2 μCi/ml of [^{3}H]thymidine for 24 hr. Pellets from the medium of JIII cultures were resuspended in 0.5 ml of 0.1 *M* Tris-HCl (pH 8.3)–0.01 *M* EGTA and centrifuged to equilibrium in a 12–60% sucrose gradient at 90,000*g* for 18 hr at 4°. Fractions (0.45 ml) were collected from the bottom of the gradients (a) 25-μl aliquots of each from the unlabeled gradient were assayed for DNA polymerase activity and expressed as ^{3}H-TMP incorporation; (b) and (c) 25-μl aliquots were assayed for acid insoluble radioactivity.

Four preparations of JIII particles were assayed for DNA polymerase after pretreatment with RNase, with and without an optimal concentration of Nonidet. RNase produced minimal inhibition of DNA polymerase in absence of Nonidet. In its presence inhibition was increased, but only partially in the range of 8–46% (Table II). The results indicate some function of the RNA in the particle on its DNA synthetic activity.

JIII particles, which synthesize DNA with endogenous templates and primers, were tested for capacity to use exogenous RNA and DNA. With exogenous heated calf-thymus DNA, the DNA synthesis of the particles was fourfold greater than the endogenous activity when synthesis was measured by ^{3}H-TMP incorporation and 23-fold greater when measured by ^{3}H-GMP incorporation. When poly(rC) oligo(dG) (the preferred synthetic RNA template for the polymerase of oncornavirions) was used, synthesis of DNA was not significantly enhanced over endogenous activity (Table III). The DNA polymerase of JIII particles, unlike polymerase of oncornavirions, does not use poly(rC) oligo(dG) under conditions where it does use exogenous DNA.

Figure 2 shows an electron micrograph of material taken from the 1.18–1.22 density region of a sucrose gradient in which a JIII tissue culture pellet had been brought to equilibrium. The material had been removed, treated with phosphotungstate and examined by electron microscopy. Only relatively uniform particles were observed, approximately 25 nm in diameter, electron luscent with a tendency to aggregate. Substructure could not be resolved.

TABLE I. Optimal Stimulation by Nonidet of DNA Polymerase Activity.[a]

Percent of Nonidet P-40 added[b]	Polymerase activity ^{3}H-TMP incorporation (cpm)
None	268
0.005	325
0.010	455
0.025	489
0.050	831
0.075	1176
0.100	1679
0.150	1092
0.200	891

[a] Twenty-five-microliter aliquots from the fraction in the 1.18–1.22 density region of the gradient with the highest enzyme activity were assayed for DNA polymerase using varying concentrations of Nonidet.

[b] Twenty-five microliters of Nonidet was preincubated with the enzyme preparation at 4° for 30 min prior to enzyme assay.

Presence of antigen(s) of extracellular JIII particles in cells derived from various species. Immune serum prepared against the particles and conjugated with fluorescein isothiocyanate-stained acetone-fixed JIII cells when tested with the direct fluorescent antibody method. The staining was generally distributed throughout the cytoplasm of the cell and was of a particulate nature (Fig. 3). The intensity of staining (1+–4+) and percentage of cells stained in each preparation varied from passage to passage, generally with a range of 10–100% of cells staining in any given preparation. Unfixed JIII cells were also stained by the conjugated JIII antiserum, indicating the presence of the antigen(s) on the surface of cells. The antiserum gave positive staining out to a 1:32 dilution.

The following experimental results determined that the staining reaction on JIII cells was specific: (a) conjugated preimmune serum did not stain the cells; (b) unconjugated immune serum gave a positive staining reaction with the indirect fluorescent antibody test using conjugated goat anti-rabbit gamma globulin, thus, indicating that globulins (antibodies) are responsible for the

TABLE II. Effect of RNase on DNA Polymerase Activity of Particles from JIII Cell Cultures.[a]

	DNA polymerase activity ^{3}H-TMP incorporation (cpm)					
	No Nonidet[b]			0.1% Nonidet		
Preparations	No RNase	RNase[b]	% Inhibition	No RNase	RNase	Inhibition (%)
1	215	188	12	746	535	28
2	815	1183	–	1120	826	26
3	448	374	16	1628	874	46
4	268	284	–	1679	1541	8

[a] Twenty-five-microliter aliquots from the fraction in the 1.18–1.22 g/cm^3 density region of the gradient with the highest enzyme activity were assayed for DNA polymerase activity with and without RNase treatment in the presence and absence of Nonidet treatment.

[b] Two-hundred micrograms of RNase and 0.1% Nonidet were preincubated with the enzyme preparation for 30 min at 4° prior to enzyme assay.

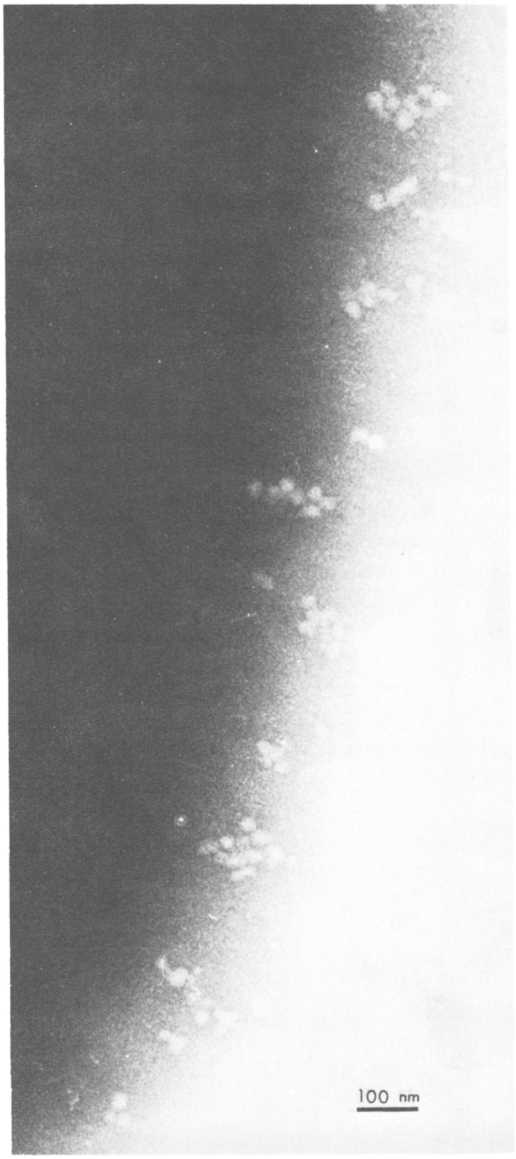

FIG. 2. Electron micrograph of particles from the 1.18–1.22 g/cm³ density region of the gradient after staining with phosphotungstic acid.

staining; (c) staining of the conjugated immune serum could be blocked completely by preincubation of JIII cells with unconjugated immune serum, but not with unconjugated preimmune serum or PBS; and (d) absorption of the conjugated immune serum with cell powder prepared from JIII cells removed the staining capacity of the conjugate, but absorption with cell powders prepared from nonmalignant human tonsilar lymphocytes had little or no effect. These results indicate that the staining observed is due to an antigen–antibody reaction in the immune serum opposed to nonspecific attachment of fluorescein dye, and that the detectable antigen is present on JIII cells but not on nonmalignant human lymphocytes.

Experiments were performed to determine if this antigen(s) on JIII cells was also present on other types of cells. Table IV gives a list of the types of cells tested with antiserum against JIII particles using the fluorescent antibody test. Only the Z-597 cell line, a continuous cell line derived from a patient with granulocytic leukemia, gave a positive staining reaction which was comparable to that observed on JIII cells. In another experiment, JIII antiserum stained both JIII and Z-597 cells at a 1:32 dilution using the direct fluorescent antibody test but specific staining characteristic of JIII cells was not observed with human fibroblasts and tonsilar lymphocytes.

Discussion. Very similar extracellular particles were detected in the culture media from two continuous cell lines (JIII and Z-597) derived from cases of human leukemia. JIII particles were related antigenically to the cells in both cultures, but not to eleven other continuous, primary, or uncultured cells derived from a wide range of species including chicken, mouse embryo, and human (Table IV). Further, human tonsilar lymphocytes failed to adsorb antibodies made against JIII particles.

While this suggests the particles may be related to the malignant aspect of the JIII cell, they do not relate to known oncornaviruses which also are released in culture from transformed cells. They resemble the oncornaviruses only in that they synthesize DNA using endogenous templates, primers, and polymerase, are stimulated by Nonidet, and are partially inhibited by RNase. They differ in density (1.18–1.22 vs 1.15–1.18), in size (25 nm vs 100 nm), in failing to use the exogenous template poly(rC) oligo(dG), and in containing some DNA as well as RNA.

The JIII particles more closely resemble certain particles of unknown significance

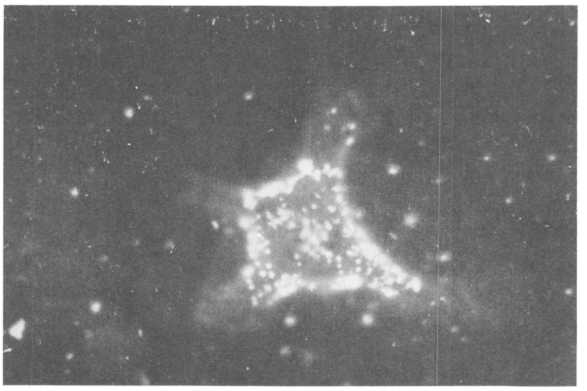

FIG. 3. Acetone fixed JIII cells stained by a 1:8 dilution of antiserum prepared against JIII particles. ×400.

TABLE III. USE OF EXOGENOUS TEMPLATES BY THE DNA POLYMERASE OF JIII PARTICLES FROM SUCROSE GRADIENTS[a].

	^{3}H-TMP incorporation (cpm)[c] Fraction number			^{3}H-dGMP incorporation (cpm) Fraction number		
Template[b]	10	11	12	10	11	12
None	147	177	164	59	85	112
DNA calf thymus	489	750	286	804	2005	559
Poly(rC) oligo(dG)	–	–	–	75	146	81

[a] Twenty-five microliter aliquots from three factions (10, 11, 12), which had the highest enzyme activity in the 1.18–1.22 density region of the gradient, were each assayed for DNA polymerase activity either with or without exogenous templates.

[b] Final concentration of templates in the enzyme reaction mixture was 20 μg/ml.

[c] All enzyme reactions were performed after preincubation with 0.1% Nonidet.

TABLE IV. STAINING REACTIONS OF DIFFERENT CELLS WHEN TESTED WITH THE JIII ANTISERUM USING THE FLUORESCENT ANTIBODY TECHNIQUE.

Cell type	Type of culture	Dilutions of antisera	Fluorescent antibody test	Staining reaction
Human leukemia (JIII)	Continuous	1:32	DF	+
		1:64	IF	+
		1:8	DU	+
Human leukemia (Z-597)	Continuous	1:8	DF	+
Green monkey kidney (Vero)	Continuous	1:4	DF	–
		1:8	DF	–
Rhesus monkey kidney ($LLCMK_2$)	Continuous	1:2	DF	–
Human cervical carcinoma (HeLa)	Continuous	1:4	DF	–
		1:8	DF	–
Chick kidney	Primary	1:2	DF	–
Human macrophages	Primary	1:2	DF	–
Baby hamster kidney (BHK21)	Continuous	1:2	DF	–
Human tonsilar lymphocytes	Not cultured	1:8	DU	±
		1:8	DU	–
Human lung (WI 38)	Diploid	1:4	DF	–
		1:8	DF	–
Rabbit Kidney (RK13)	Continuous	1:2	DF	–
Human fibroblasts	Diploid	1:4	DF	±
		1:8	DF	–
NIH Swiss mouse embryo	Primary	1:8	DF	–
		1:4	IF	–

Note: DF, direct fluorescent antibody method on fixed cells; IF, indirect fluorescent antibody method on fixed cells; DU, direct fluorescent antibody method on unfixed cells.

previously detected in medium from a variety of cultured malignant cells and in certain human plasma. Particles with a density of 1.15–1.21 containing RNA and DNA (like JIII particles) were detected by Long *et al.* (6) in the medium of cultured spleen cells obtained from a patient with Hodgkin's Disease. Narayan and Rounds (7) detected 10–12 nm ring shaped particles in medium from cultured human adenocarcinoma cells. These also contained both RNA and DNA but had a density (1.6–1.19) similar to oncornaviruses. The plasma of two patients with chronic lymphocytic leukemia yielded to Kiessling *et al.* (8) particles with properties common to JIII particles. Both particles had similar densities and contained a DNA polymerase, which was stimulated by Nonidet and by exogenous DNA but not by synthetic RNA templates, poly(rC) oligo(dG) (JIII particles), and poly(rI-rC) (Kiessling's particles).

The release of complex particulates from cells in culture might be viewed as an aberration of some normal (albeit unknown) function of the cell or an additional example of a cytopathology induced by an unknown virus.

The development of a fluorescent antibody against one or more antigens of the particulate allows use of the antigen as a marker to learn if the particle can be replicated in those cells in which the antigen is presently absent. Information concerning the distribution of the antigen(s) in various pathologic human states might also be meaningful in establishing the significance of the particle. How fluorescent antibody might also facilitate collection of such relevant data will be illustrated in a separate communication.

Summary. Extracellular particles, with a density of 1.18–1.22 g/cm^3 in sucrose, were detected in the culture medium of a continuous cell line (JIII) derived from a patient with monocytic leukemia. These particles contained RNA, DNA, and a DNA polymerase. They synthesized DNA with endogenous templates and primers and also used exogenous DNA but not poly(rC) oligo(dG) as a template. Pretreatment with Nonidet P-40 stimulated DNA polymerase activity while treatment with ribonuclease partially inhibited the enzyme activity. Fluorescent antibodies made to the particles stained both JIII and Z-597 cells derived from human leukemias but not other types of human or nonhuman cultured cells tested. The particles do not appear to be oncornaviruses but may be a particulate antigen associated with malignant cells of hemopoietic and lymphoid origin.

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