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Biological Activation of Virus-Like Particles from Chemically Induced Guinea Pig Sarcomas[1] (38885)

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(Introduced by John M. Adams)

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Virus-like particles have been observed in oogonia and oocytes of fetal, neonatal, and adult albino guinea pigs (1). Moreover, reports from many laboratories indicate that virus-like particles have been activated from cultured cells of leukemic and normal guinea pigs by chemical treatments (2–6). Such particles have also been described in cells of 3-methyl-cholanthrene (MCA)-induced guinea pig tumor cells after induction by 5-bromodeoxyuridine (5).

We recently observed particles resembling viruses in three different types of MCA-induced guinea pig sarcomas that had morphological, biochemical, and biophysical properties similar to each other and to the virus-like particles found in tumors induced by known oncornaviruses (7).

We report the induction of similar particles by biological activation of the guinea pig sarcoma cells with adenovirus and chemical treatments. We report the biochemical, biophysical, and immunovirological properties of the particles found in both non-activated and activated cultures.

Materials and Methods. Guinea pigs. Adult Sewall Wright, strain 2 female guinea pigs were used in this study. This strain has been maintained by continuous single line brother–sister mating. First- and second-set skin grafts between members of this strain are permanently accepted (8).

Tumors. Tumors investigated in this study were of three distinct histologic types: MCA-25, an osteogenic sarcoma; MCA-A, a liposarcoma; and MCA-1, a fibrosarcoma. Each was induced by subcutaneous injections of MCA, in female strain 2 guinea pigs. The histopathology, immunogenicity, and *in vivo* growth characteristics of MCA-induced guinea pig sarcoma tumors have been previously described (9–11). Tumors were maintained by dorsal subcutaneous injection of 1 ml of a 30% suspension (v/v) of minced tumor in Eagle's minimal essential medium (MEM) unsupplemented. Earlier tumor transplant generations were preserved by slow freezing in 10% dimethylsulfoxide in RPMI 1640 with 40% fetal bovine serum (FBS) and storage at −190°.

Sera. Serum specimens were collected by standard methods and stored at −70° in 1-ml quantities. Serum blood specimens were obtained from breeder animals, from animals with growing tumors, from animals in which the growing tumors had been excised, from animals prior to tumor inoculation, and from animals immunized by temporary tumor growth after subsequent tumor challenge.

Cell cultures and media. MCA-25, MCA-A, and MCA-1 tumors were excised, minced into fine bits with Mayo scissors, washed in sterile physiologic saline, and then dispersed into single cell suspensions with 0.25% trypsin (Crystalline Trypsin, Difco Co.) After the addition of 10 ml of FBS to the suspension to stop digestion, the cells were centrifuged at 400*g*, washed two times in Hanks' solution and then resuspended in Prehn's MEM with 20% FBS, 1% each of penicillin and streptomycin, and 40 mg/liter of gentamycin. Plastic flasks (T75, Falcon Plastics, Oxnard, CA) were inoculated with 4×10^6 cells in 15 ml of medium. The flasks were incubated at 37° in a humid incubator in 5% CO_2. Confluent monolayers were usually established within 1 wk for the MCA-A and MCA-25 cells and

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within 2 wk for MCA-1 cells. Media was changed every 2–3 days and supernatants were frozen at −70° after collection. Activation studies were started when cells reached 70% or more confluency. During and after activations, cells were maintained on Dulbecco's MEM with 0.5–10% FBS, 2 m*M* glutamine, 100 U/ml penicillin, 50 μg/ml streptomycin, and 5 μg/ml fungizone.

Immunization by temporary tumor growth. Single cell suspensions prepared by enzymatic digestion were inoculated into strain 2 guinea pigs at approximately 1×10^6–1×10^7 viable tumor cells as previously described (9). After 2 wk the tumor nodules were excised and animals were challenged with 10^7 cells 10 days postexcision. No immunized animals developed tumors after challenge.

Electron microscopy. Small pieces (approximately 1 mm^3) of fresh tumor and normal tissues were fixed in 2.5% glutaraldehyde in 0.01 *M* phosphate buffer, stained with lead citrate–uranylacetate, and then processed according to previously described methods (9). A Phillips 200 electron microscope was used to study the specimens.

Concentration of culture fluids. Concanavalin A (Con A) was used to agglutinate virus-like particles from culture medium of induced and uninduced cultured cells by the methods of Stewart and Maizel (12) and Stewart *et al.* (13).

Arginine-deprivation treatment. Growth medium was decanted from cells in primary and later passage cultures, the cells were washed twice in DMEM without arginine or serum and then incubated in this medium for 4 hr. The culture fluids were again decanted and fresh DMEM without arginine, but with 10% FBS, was added to the cultures.

Chemicals and isotopes. The chemical used was 5′ bromo-2-deoxyuridine (BrdU) obtained from Calbiochem, Los Angeles, CA. ^{3}H-uridine, ^{3}H-thymidine, and polynucleotides were purchased from Schwartz/Mann, Orangeburg, NY. Oligonucleotides were obtained from P-L Biochemicals, Inc., Milwaukee, WI.

Adenovirus. Human adenovirus type 5 (Ad-5) was grown in a cultured human tumor cell line ONSA-8 (14) and purified on CsCl density gradients as previously described (15). Plaque assays on human embryonic and ONSA-8 cells were used to ascertain Ad-5 infection. Plaques were counted 10–16 days post-virus inoculation of cell cultures (16).

Purification of virus-like particles. Cell-free fluids concentrated by Con A and extracts of cells processed as described by Sarngadharan *et al.* (17) were purified by sucrose velocity gradients followed by equilibrium sucrose density gradients.

Activation by adenovirus type 5. Cell cultures of the guinea pig sarcomas at 70–80% confluency were decanted of their medium and then inoculated with Ad-5 at concentrations of 5, 50, or 500 pfu/cell in fresh unsupplemented DMEM. After 24- to 48-hr incubation at 37°, the inoculum was removed and the cell cultures were washed twice in DMEM with 0.5% FBS and reincubated. Replicate cultures received ^{3}H-uridine or ^{3}H-thymidine or remained without isotope during the postinoculation period. Cells and supernatants were collected at various intervals and immediately assayed for infectious adenovirus, for isotope incorporation into DNA and RNA, and for DNA polymerase reverse-transcriptase activities.

Biological assays. Cell-free filtrates of culture medium, extracts of uninduced guinea pig sarcoma cells, and fractions from sucrose gradients with peaks of radioactive ^{3}H-uridine from activated cultures were assayed in mixed-culture cytopathogenicity tests. Host range assays for Type C viruses were performed on Balb/c 3Tc cells with XC assays (18) and for N-trophic virus on NIH Swiss 3T3 cells with XC assays (19) by Dr. Esther Hayes. Cytopathology of the guinea pig materials was assayed on adult embryonic and fetal human cell cultures.

Immunodiffusion. Microimmuno-double-diffusion tests were performed and conditions for maximum immunoprecipitation were established as previously described (20).

Activation by BrdU. Cell cultures at 80–90% confluency were decanted of their growth medium and then inoculated with

50, 100, 150, or 200 μg/ml of BrdU in DMEM containing 0.5% FBS. Chemical treatment continued for 24–72 hr. Medium was changed to one without chemical and the cells were incubated for another 6–8 days. Cell-free fluids, after Con A concentration, were examined for DNA polymerase activity and ^{3}H-uridine and ^{3}H-thymidine labeling. ^{3}H-Isotopes were added to cultures after 24 hr of chemical treatment.

Radioisotope labeling of cultures. Incorporation of ^{3}H-uridine into RNA and ^{3}H-thymidine into DNA was accomplished by adding isotopes to individual cultures at a final concentration of 20 μCi/ml in fresh DMEM with 0.5% FBS.

Assay of DNA polymerase activity. Supernatant fluids (after rate and equilibrium sucrose density-gradient purification and pelleting) were assayed for DNA polymerase activities to detect RNA-dependent DNA polymerase. Synthetic templates, poly rA: oligo dT, poly dA: oligo dT and poly rC: oligo dG, were used to increase enzyme detection. Assays were performed according to methods described by Sarngadharan *et al.* (17) with these modifications: Polymerase assays used 50 μl test sample (enzyme) plus 50 μl standard reaction mixture plus 10 μl synthetic template. Standard reaction mixture for use with poly rA: oligo dT and poly dA: oligo dT templates contained 10 μl 1 *M* $MgCl_2$, 10 μl 1 *M* dTT, 40 μl 1% TX-100, 50 μl 1 *M* NaCl. 100 μl Tris–HCl, pH 8.3, dATP, dCTP, and dGTP at 15.6 μ*M*/liter and ^{3}H-TTP at 17 Ci/mmole. ^{32}P-TTP was used alternatively to confirm that the ^{3}H-uridine peak materials also had polymerase activity. In reactions using poly rC: oligo dG, TTP and ^{3}H-GTP were appropriately substituted to the over-all reaction mixture. Reactions proceeded for 60 min, then were stopped by the addition of 10% TCA containing 0.02 *M* sodium pyrophosphate. Materials were collected on Millipore filters and counted on LS-250 scintillation counter (Beckman; ^{3}H-counting efficiency 60%). Enzyme samples were pretreated prior to assay with NP-40 at 0.01–0.05% for 30 min in an ice bath (4°).

Results. Spontaneous release of virus-like particles. Primary cell cultures of each of the MCA-induced guinea pig sarcomas were incubated over a 20-day period with com-

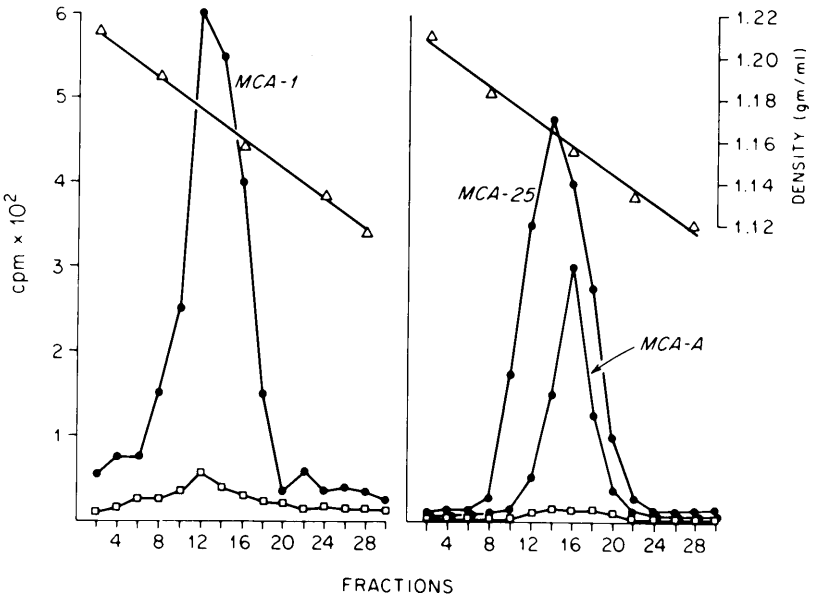


FIG. 1. Equilibrium sucrose density-gradient analysis of purified ^{3}H-labeled guinea pig sarcoma particles [^{3}H-uridine (●—) and ^{3}H-thymidine (□—)]. ^{3}H-Uridine peaks at 1.16–1.18 g/ml showed light-scattering bands in all three types of sarcoma (MCA-1, MCA-25, and MCA-A). △ represents sucrose density measurement.

plete medium changes each 7 days. One-third of the cultures were pulse labeled with ^{3}H-thymidine, one-third with ^{3}H-uridine, and one-third were unlabeled. Cells were labeled for 24–36 hr before fluids were collected for assay. Cell-free fluids from the unlabeled cultures were assayed for DNA polymerase activity. Likewise, fluids from the thymidine- and uridine-labeled cultures were assayed for peaks of radioactivity. Virus-like particles were isolated from all cell-free fluids by ultracentrifugation on serial sucrose density gradients.

Peaks of radioactivity were observed in supernatants from cultures of all three sarcomas types labeled with ^{3}H-uridine at 1.16–1.18 g/ml on final equilibrium sucrose density gradients (Fig. 1). No significant radioactivity was detected from ^{3}H-thymidine-labeled cultures in similar equilibrium sucrose gradients. Maximum radioactivity in labeled peak materials was found at 12 days with all three types of sarcomas. No activity was observed at 4 days; low, but detectable, activity was found at 8 days; and less than the maximum activity at 16 and 20 days was seen, using the same conditions of labeling, cell cultures, initial cell numbers per culture, and the amount of fluid assayed. Fluids from MCA-1 sarcoma cultures at 12 days contained the highest amounts of ^{3}H-uridine materials at sucrose densities of 1.16–1.18 g/ml (Fig. 1).

Chemical induction of cultured guinea pig sarcoma cells. Primary cultures of the three types of guinea pig sarcoma cells, 8 days old, were exposed to increasing doses of BrdU. The cells remained morphologically unchanged after exposure to doses of 25 and 50 μg/ml. However, at higher chemical concentrations of 100, 150, and 200 μg/ml, the cells became less distinct at the borders, and showed increased granularity. Cell growth was affected at all concentrations of drug tested although the highest doses of 150 and 200 μg/ml caused cessation of growth at approximately 3 days posttreatment. Cultures were treated initially for 72 hr, but as the effects on cell growth and morphology were observed the treatment period was shortened to 48 hr.

Observed DNA polymerase activity in the supernatants increased significantly after chemical treatment with BrdU (Table I). Maximum production or release of the DNA polymerase-active materials occurred at 6 days after chemical treatment. Continued cultivation of cells in BrdU medium or refeeding the cell cultures already treated at 3-day intervals with medium containing fresh BrdU did not enhance the DNA polymerase activities, although the activity was always detectable at levels well above those of untreated cultures.

Activation by arginine deprivation. After growth medium was decanted, confluent monolayers of primary cell were washed twice and incubated for 4 hr in medium without serum or arginine to deplete the normal arginine levels. Cultures were refed with arginine-deficient medium with serum and incubated. Fluids were collected at 3-day intervals for 15 days. DNA polymerase activity was detected in concentrated culture fluids from arginine-deficient cultures although there was no indication

TABLE I. RADIOACTIVITY AND DNA POLYMERASE ACTIVITY (IN FLUIDS) FROM BRDU-INDUCED GUINEA PIG SARCOMA CULTURES.

Tumor cells	BrdU (mg/ml) treatment	^{3}H-uridine activity [(cpm/ml; density (g/ml)]		DNA polymerase activity Poly rA: oligo dT template
MCA-25	None	550	1.16–1.18	158[a]
	BrdU 150 (48 hr)	8300		873
MCA-A	None	430	1.16–1.18	233
	BrdU 150 (48 hr)	3150		680

[a] Counts per minute of ^{3}H-TMP incorporated per ml. Templated reactions using poly dA: oligo dT did not exceed 280 cpm.

TABLE II. DNA POLYMERASE AND RADIOACTIVITY IN FLUIDS OF GUINEA PIG SARCOMA CULTURES ACTIVATED BY ARGININE DEPRIVATION.

Tumor cells	Treatments	^{3}H-Uridine activity [cpm/ml; density (g/ml)]		DNA polymerase activity with templates Poly rA:oligo dT	Poly dA:oligo dT
MCA-25	None	675	1.16–1.18	515	88
	Arginine deprived	3550		525	92
MCA-A	None	530	1.16–1.18	1050	153
	Arginine deprived	3220		1150	145

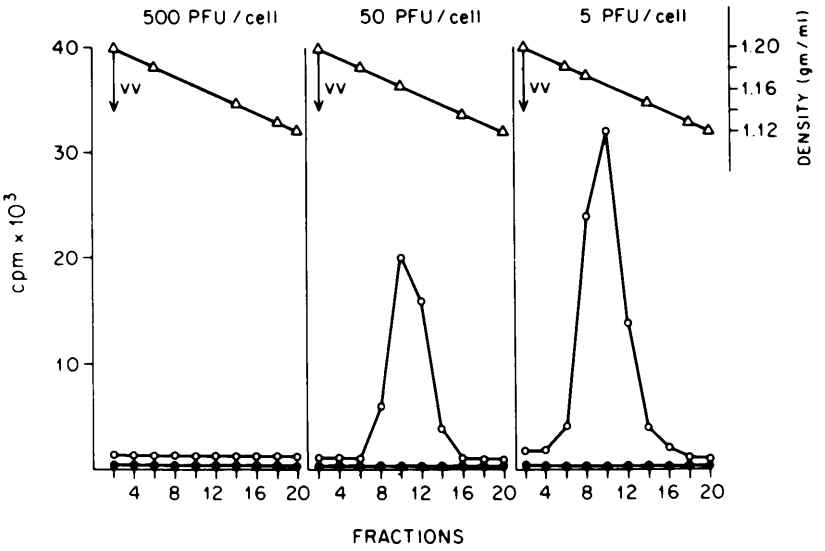


FIG. 2. Radioactivity profiles [^{3}H-uridine (○—) and ^{3}H-thymidine (●—)] after equilibrium sucrose density-gradient analysis of virus-like particles activated from MCA-25 guinea pig sarcoma cultures. VV represents unlabeled AD 5 used as a visible marker.

of any significant increase in this activity when compared to the cultures in complete medium (Table II). An increase in ^{3}H-uridine, but not ^{3}H-thymidine, activity in the 1.16- to 1.18-g/ml fractions of equilibrium density sucrose gradients was observed in the arginine-deficient culture fluids.

Biological induction of guinea pig sarcoma cells by using human adenovirus type 5. Primary cell cultures of guinea pig sarcomas MCA-A and MCA-25 were inoculated in groups of six, respectively with 5, 50, or 500 PFU per cell of CsCl gradient-purified human adenovirus type 5 (AD 5) in DMEM with 0.5% FBS. After 24 hr of incubation, the inoculum was removed, the cells were washed with fresh unsupplemented medium, and refed with fresh DMEM with 5% FBS. Supernatants were collected for 8 days after inoculation.

Supernatants from cultures inoculated with 500 PFU of adenovirus type 5 (AD 5) showed that both RNA and DNA synthesis in these cultures had apparently been reduced to almost nil values. The cells of these cultures also changed morphologically over the 8 days from spindle-shaped cells to tightly packed, rounded cells with indistinct cell margins as observed by phase microscopy. None of the typical early cytopathic effects of adenovirus infection were observed in these cultures.

In contrast, fluids from cell cultures exposed to 5 and 50 PFU, respectively, of AD 5 showed increasing levels of ^{3}H-uridine after sucrose density equilibrium gradient analysis. Supernatants of ^{3}H-thymidine-labeled cultures showed no peak of activity by similar gradient analysis (Fig. 2). Supernatants from unlabeled cultures inoculated

TABLE III. RADIOACTIVITY AND DNA POLYMERASE ACTIVITY (IN FLUIDS) FROM GUINEA PIG SARCOMA CULTURES ACTIVATED BY ADENOVIRUS TYPE 5.

Tumor cells	Treatments (pfu/cell)	^{3}H-Uridine	^{3}H-Thymidine peaks (cpm/ml)	Density (g/ml)	DNA polymerase activity Poly rA:oligo dT
MCA-25	None	557[a]	375[a]		180[b]
	AD5(500)	633	508	1.16–1.18	85
	AD5(50)	19,573	468		1,200
	AD5(5)	28,070	645		1,900
	AD5(5, 50, 500)	210[c]	135[c]	1.20–1.24	78[c]

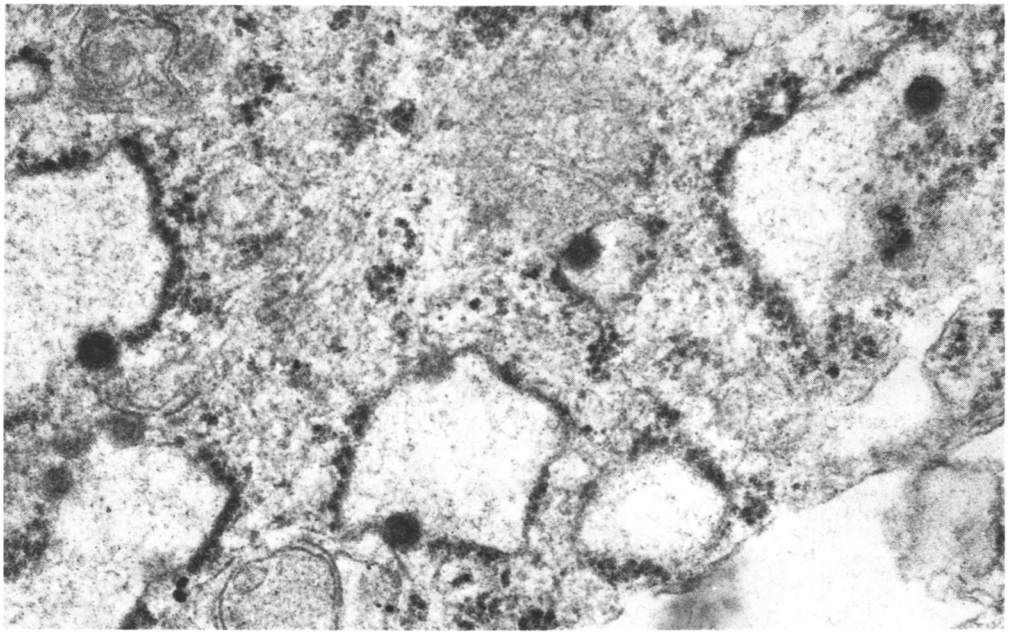
[a] Counts per minute per ml of peak at designated density after equilibrium sucrose density gradient.
[b] Counts per minute of ^{3}H-TMP incorporated per ml.
[c] Mean of counts per minute detected in three experiments of cultures infected with the different doses of AD 5.

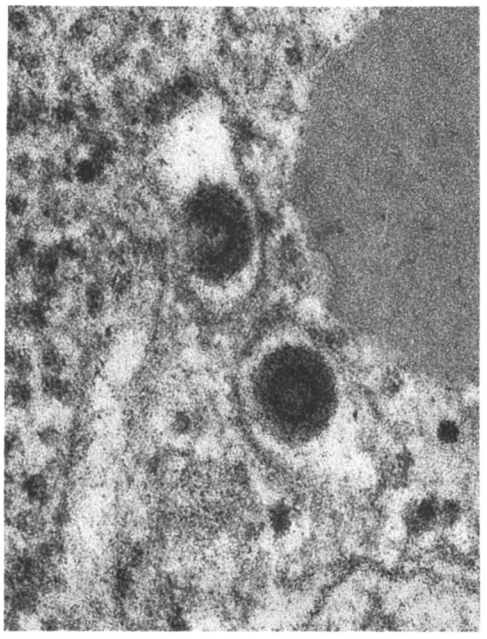
with 5 and 50 PFU/cell of AD5 contained DNA polymerase activities at increased levels, whereas little activity was detected from fluids of cultures inoculated with 500 PFU/cell (Table III). No evidence of AD 5 replication was observed in the cells or supernatant fluids of the inoculated cultures by radioactivity by infectious virus assays or by electron microscopic observations of the inoculated cells. Results of our attempts to induce endogenous virus activities from nontumor guinea pig tissues have shown little or no response to adenovirus activation.

Morphology of the particles in cultured MCA-induced guinea pig sarcomas. Intracisternal particles resembling type A particles were most commonly observed in treated and untreated cells by electron microscopy (Figs. 3 and 4). Extracellular particles were not observed in untreated cells and were extremely rare in treated cells. Intracytoplasmic particles were observed infrequently, and these particles seemed to be budding from cell surfaces. Particles were approximately 90–100 nm in diameter, had a regular, thick coat, and a central nucleoid (Fig. 5). No herpesvirus-like particles were observed in any of the guinea pig sarcoma cells during serial *in vivo* or *in vitro* passage.

Immunological relationships of the guinea pig sarcoma-induced particles. Fluids from BrdU and adenovirus-activated sarcoma cultures were individually pooled. Fractions from the 1.16- to 1.18-g/ml densities of equilibrium sucrose density gradients were dialyzed against unsupplemented medium for 12 hr, phosphate-buffered saline for 4 hr, concentrated to 10–100 of original volume and then disrupted by sonication and Tween–ether. These materials did not react in Ouchterlony-type immunodiffusion tests with commercially prepared antisera to murine, rat, and feline leukemia–sarcoma viruses or with serum specimens from strain 2 guinea pigs used as breeder stocks. However, all serums from guinea pigs that had active tumors, that had had tumors resected, and that had been injected with extracts of the sarcoma cells reacted positively with the disrupted guinea pig sarcoma virus-like particles derived by either type of activation. All positive serums demonstrated common precipitating antibodies. Attempts to grow the activated virus-like particles in human and cavian cells failed and negative results were obtained in host range assays for B- and N-trophic viruses in XC assays (E. Hayes, personal communication).

Discussion. Results of our investigations show that virus-like particles that have RNA-dependent DNA polymerase activities can be activated from cultured cells of three different histologic guinea pig MCA-induced sarcomas. Chemical activations of virus particles using BrdU were successful in all guinea pig tumor cultures and our results support those found in previous studies of various types of guinea pig cultured cells. (2–6). In contrast, arginine deprivation as a means of activation of virus particles was not successful in our hands, although this





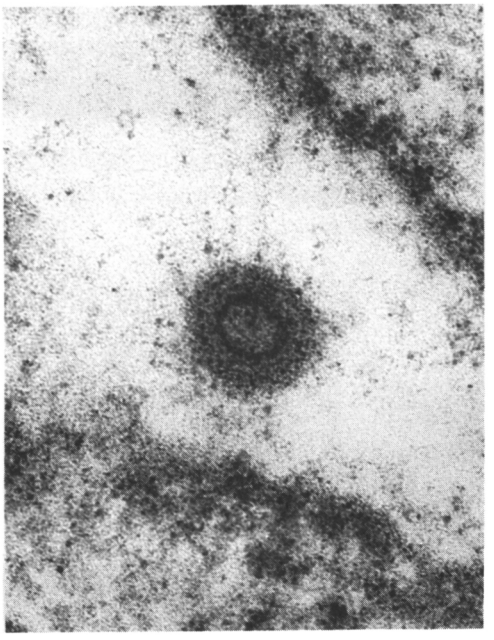


FIG. 3. Intracisternal particles seen in unactivated guinea pig liposarcoma cells. (×59,000).

FIG. 4. Particles observed to be budding into and contained in cytoplasmic vacuoles of AD 5 activated guinea pig osteogenic sarcoma cells. (×122,000).

FIG. 5. Particle appearing to be in cytoplasm of BrdU-treated guinea pig fibrosarcoma cells. Particle location my be result of plane of sectioning. (×185,000).

method has been used for activation of viruses from RSV-transformed rat cells and from human leukemic cells by Kotler *et al.* (21, 22). We found that virus-like particles (VLP) could be induced in the MCA guinea pig sarcoma cell cultures by inoculation with active human adenovirus type 5, but not with inactivated virions.

Because RNA VLP were activated by a human DNA virus, it would appear that the DNA virus performs some function either in the host cell or in the sequence of RNA virus assembly to produce or release endogenous VLP. One role for a DNA virus in this system might be to induce VLP in a similar way as BrdU or other chemical activators by influencing the cellular DNA. Thus, both chemical and biological induction methods may ultimately have the same result and virus release may be simply a function of cell degeneration.

Although we did not observe herpesvirus-like particles in association with the guinea pig particles in our guinea pig sarcoma cells, both of these types of virus particles—one DNA and the other RNA—have been reported in guinea pig cells (24, 25). Furthermore, consecutive activation of a type C RNA and the DNA polyoma virus from cultures of tumors induced by polyoma-transformed rat cells has been recently reported (28). In addition, pseudo-type formation between vesicular stomatitis virus, an RNA virus, and herpes simplex, a DNA virus, has been described (23).

Our previous observations of adenovirus activities in cultured cells of developing mouse embryos demonstrated rapid virus attachment and penetration (26) and suggested that endogenous murine virus particles in the cells were produced at higher levels in the adenovirus inoculated cultures than in untreated control cultures (Winters and Chase, unpublished data).

Our observations show that guinea pig sarcoma cells cultured *in vitro* spontaneously release VLP at low levels.

Furthermore, serums from guinea pigs with MCA-induced sarcomas demonstrated common precipitating antibodies to disrupted guinea pig VLP derived by our activations. These results suggest that virus particles and virion subunits *in vivo* might be readily available to act as strong stimulators of the animal's humoral and cellular immune responses. Indeed, immunological cross reactions observed by some investigators studying the humoral- and cell-mediated immune responses in chemically induced guinea pig systems and, perhaps, in other similar animal models, may have been due to immunogenic effects of the endogenous viruses that were being assembled at or released from cell surfaces (9, 10).

Our results concur with other previous observations that primary cultures are superior to later subcultures in demonstrating VLP in guinea pig systems (5). The morphology of particles in the guinea pig cells is still unresolved. We see type A particles, others have seen mostly type C (27) or type A and type C (4). Recently, type B particles were reported together with some type A particles (5, 6). No type B particles have been observed in our cells, untreated or after activation with either chemicals or adenovirus. We are attempting to extend our biological activation techniques to other systems and to understand the underlying mechanisms.

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