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Induction of Guinea Pig Leukemia-Like Virus from Cultured Guinea Pig Cells[1] (38335)

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Viral particles have been observed in tissues taken from leukemic guinea pigs and in normal guinea pigs (T 1–4). However, the putative guinea pig leukemia virus had not been isolated in tissue culture. It has recently been reported that virus particles considered to be morphologically similar to type C virus particles can be activated after 5-bromodeoxyuridine (BrdU) treatment of cultured cells from leukemic and normal guinea pigs (5, 6).

We have recently described activation of particles resembling guinea pig leukemia virus from guinea pig nonproducer (NP) clone No. 12 cells by treatment with BrdU (7). We now report induction of similar particles not only from guinea pig NP cells but also from normal guinea pig cells after BrdU treatment. The detailed chemical induction of guinea pig leukemia-like virus from cultured guinea pig cells and further characteristics of the virus and some of its biophysical, serological, and biological properties are also described.

Materials and Methods. Cell cultures and media. Guinea pig cells were prepared from the NIH strain and strain 13 (obtained from the animal production of National Institutes of Health). Cells were grown and maintained in Eagle's minimal essential medium with 10% fetal bovine serum, 2 m*M* glutamine, 100 unit/ml penicillin, and 100 μg/ml streptomycin (EMEM + 10% FBS).

The guinea pig (NIH strain) NP clonal lines (8) used in this study were isolated from transformed foci induced by the Kirsten strain of murine sarcoma virus (Ki-MSV) (9) and have been described in detail elsewhere (8). Such NP cells did not produce infectious virus detectable by focus assay (10) or complement-fixation (CF) tests for murine leukemia virus (COMUL test) (11, 12) in mouse, rat, or guinea pig cells. However, they were morphologically indistinguishable from virus-releasing MSV-transformed GPE lines and produced tumors when transplanted into newborn guinea pigs. No RNA-dependent DNA polymerase activity was found in these lines. Also, no virus particles of specific gravity of approximately 1.15 g/ml could be found in the fluids of these cultures after incubation with [^{3}H]-uridine and isopycnic centrifugation on sucrose density gradients. However, the sarcoma virus genome could be rescued in these NP cells by cocultivation with a "helper" murine leukemia virus-releasing GPE cells. In addition, neither virus nor virus-like particles were observed in these cells when examined by electron microscope.

The AD 023D line (normal hybrid guinea pig embryo fibroblast cells) was kindly supplied by Dr. G. D. Hsiung, Veterans Administration Hospital, West Haven, Conn. A chemically induced guinea pig tumor line was a gift from Dr. I. Green, National Institutes of Health, Bethesda, MD. The guinea pig tumors induced by Ki-MSV (Rhim and Green, unpublished) were also used.

Chemicals. Chemicals used were 5-bromodeoxyuridine (BrdU), 5-iododeoxyuridine (IdU), 5-fluorouracil, 5-fluorodeoxyuridine, and 2-amino-6-mercaptopurine (Calbiochem, Los Angeles, CA), 3-methylcholanthrene (3MC), 7,12-dimethylbenz(*a*)anthracene (DMBA)

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(Eastman Organic Chemicals, Rochester, NY). Cytocholasin B was obtained from Imperial Chemical Industries Research Labs, Cheshire, England. All the chemicals were first dissolved in distilled water to yield a stock solution of 1000 μg/ml, which were stored at 4°. The desired concentration was prepared by diluting stock solution with the medium (EMEM + 10% FBS). The solution of 3MC, DMBA, and cytocholasin B was made in the same way except that acetone was used as diluent for their stock preparation.

Induction of virus by chemical treatment. One-day-old cultures of normal or NP cells were usually exposed to various doses of chemicals for 1 day. After treatment of chemicals cells were washed and fresh medium was added. At various intervals, fluids and cells were examined for the presence of reverse transcriptase activity, [^{3}H]-uridine-labeled virus, and guinea pig leukemia-like particles.

Virus assay. A combination of the following procedures was used to detect the replication of virus in cultures: (1) the *in vitro* COMUL test; (2) RNA-directed DNA polymerase assay; (3) [^{3}H]-uridine-incorporation test; (4) electron microscopy examination.

Complement fixation. Complement-fixation tests were carried out in the microtiter technique described for tumor antigen studies (13). Titers were recorded as reciprocals of the highest dilution giving 3+ to 4+ fixation of 1.8 units of complement.

Preparation of CF antigens. Cell pack preparation for CF testing were made as previously described (11).

Electron microscopy. Preparation and examination of cells by electron microscopy were carried out as previously described (14).

Assay of reverse transcriptase activity. The procedure of assaying supernatant viral RNA-dependent DNA polymerase, which is a highly sensitive method for the detection of replicating type C RNA virus, has been described in detail (14). A synthetic template, poly (rA:oligo dT), was used to enhance enzyme detection.

[^{3}H]-uridine incorporation. Incorporation of [^{3}H]-uridine into viral RNA was as follows: [^{3}H]-uridine was added to freshly seeded cultures to give a final concentration of 20 μCi/ml. The culture medium was harvested at 48 hr and the presence of newly synthesized labeled virus was determined by sucrose density-gradient fractionation of clarified culture fluid and examination of the radioactivity of the fractions.

Results. The effect of BrdU on cultured guinea pig cells. One-day-old cultures of normal guinea pig (NIH strain and Strain 13) cells and NP clonal lines (No. 12 and No. 2) were exposed to various doses of BrdU. All the cells grew well after BrdU treatment. Time course of induction of reverse transcriptase in culture fluids of guinea pig cells after exposure to various BrdU doses for 24 hr is shown in Table I. Activation of reverse transcriptase was observed not only in NP clonal cells but also in cultured, normal guinea pig cells from embryo, kidney, and spleen. There was no evidence of polymerase activity in supernatants of untreated cultures. However, by day 4, a small amount of enzyme activity was observed in the treated cultures. Polymerase activity in supernatants reached a maximum between 6–8 days after the cells were exposed; but by 14 days after exposure, polymerase was barely or not detectable. Maximum induction was usually obtained at day 8 after exposure to 25–50 μg BrdU/ml for 1 day (Table I). Prolonged cultivation in the BrdU-containing medium for 4–8 days did not increase the polymerase titer. However, polymerase activity was always detectable in the BrdU-containing medium of the culture in prolonged cultivation (Table II). Polymerase activity was also induced after exposure of virus or chemical-induced guinea pig tumor cells to BrdU. In tumor cells, better production of enzyme activity was always observed in the BrdU-containing medium in prolonged cultivation at the dose tested (Table II).

The reinducibility of virus from guinea pig cells by BrdU. The very fact that the induced reverse transcriptase activity did not last longer than 2 wk in the treated cells, we were interested in knowing whether these treated cells were reinducible. By splitting the previously treated cells into two flasks, one of them was exposed again to BrdU (25 μg/ml) for 24 hr, the other was kept in growth medium only. Six days after exposure supernatant fluids from each culture were harvested and assayed for viral enzyme activity as described above. The results were shown in Table III. No matter what cell lines were used, induction was always observed. The induction was transient and BrdU dependent. As far as the nonproducer clone No. 12 cell is concerned, this mode of action has not been changed for at least seven passages. The difference be-

TABLE I. Activation of Reverse Transcriptase and Particles Resembling Guinea Pig Leukemia Virus in Cultured Guinea Pig Cells After BrdU Treatment.[a]

Group	Cells	BrdU (μg/ml) treatment	Reverse transcriptase activity at the day after exposure 4	6	8	14	Guinea pig leukemia-like particles
Random-bred guinea pig NIH strain	Embryo (P-1)	None	73[b]	62	64	88	Negative
		BrdU 25 (1 day)[c]	140	630	688	92	Positive
	Kidney (P-1)	None	73	61	58	72	Negative
		BrdU 25 (1 day)	251	406	162	114	Positive
		BrdU 50 (1 day)	—[d]	350	216	—	—
	Spleen (P-1)	None	—	67	58	63	Negative
		BrdU 25 (1 day)	—	265	451	128	Positive
		BrdU 50 (1 day)	—	181	148	—	—
	Nonproducer Clone No. 12 (P-19)	None	104	83	92	64	Negative
		BrdU 25 (1 day)	306	564	950	99	Positive
		BrdU 50 (1 day)	475	716	812	—	Positive
		BrdU 100 (1 day)	386	752	688	—	—
		BrdU 200 (1 day)	265	672	684	—	—
		None	64	72	66	—	Negative
		BrdU 25 (1 day)	353	688	909	—	Positive
		BrdU 25 (8 day)	250	560	650	—	—
	Nonproducer Clone No. 2 (P-11)	None	—	70	—	66	Negative
		BrdU 25 (1 day)	—	345	—	72	Positive
Inbred Guinea pig Strain 13	Embryo (P-4)	None	—	66	—	—	Negative
		BrdU 25 (1 day)	—	350	—	—	Positive
	Kidney (P-1)	None	—	74	—	—	Negative
		BrdU 25 (1 day)	—	320	—	—	Positive

[a] After treatment of BrdU cells were washed and fresh medium was added. At various intervals the medium was collected and examined for viral enzyme as described. Cells were also examined for the presence of particles between 8 and 10 days after treatment.

[b] Counts per minute of incorporated [^{3}H]TMP per ml.

[c] Duration of treatment.

[d] Not done.

tween treated and untreated cells at each passage is obvious. Morphologically, these re-treated cells became more heterogeneous than their mother population. Giant and flatten cells were seen more frequently. On the other hand, there was no significant change in their growth rate.

Effect of various chemicals on guinea pig nonproducer cells. Various chemical compounds were tested for their ability to induce reverse transcriptase activity from NP clone No. 12 cells (Table IV). Among these chemicals at the dose tested, IdU and BrdU were the most effective ones in inducing reverse transcriptase activity. 5-fluorourinal, 2-amino-6-mercaptopurine, and 3-methylcholanthrene also caused moderate or little increase in this enzyme activity. On the other hand, cytochalasin B and 7,12-dimethylbenz(a)anthracene had little effect. The peak activity was demonstrated 7 days after the 1-day BrdU or IdU exposure. The effect of induction had completely disappeared 14 days after exposure. The present findings were similar to those of Teich *et al.* (15) and Aaronson (16) for activation of viruses by chemicals; IdU and BrdU were superior to all other compounds tested.

Morphology of BrdU-activated particles from cultured normal guinea pig cells. Particles resembling guinea pig leukemia virus observed in the BrdU treatment NP No. 12 cells (8) were not

TABLE II. Reverse Transcriptase Activity in the BrdU-Containing Medium of Cultured Guinea Pig Cells.

Cells	BrdU (μg/ml) treatment	Reverse transcriptase activity at the day after exposure				
		6	12	19	26	33
Nonproducer	None	72[a]	84	116	94	87
Clone No. 12	BrdU 25 (1 day)	565	144	125	93	86
NIH strain (P-25)	BrdU 25 (all the time)	450	336	748	711	349
Hybrid guinea pig	None	105	114	—[b]	—	—
Embryo AD 023D	BrdU 25 (1 day)	853	220	—	—	—
(P-4)	BrdU 25 (2 day)	431	274	—	—	—
	BrdU 25 (all the time)	671	270	—	—	—
Strain 13	None	44	—	—	—	—
Ki-MSV induced	BrdU 25 (1 day)	105	—	—	—	—
Tumor cells	BrdU 50 (1 day)	115	—	—	—	—
(P-5)	BrdU 100 (1 day)	160	—	—	—	—
Strain 2	None	108	82	72	—	—
Ki-MSV induced	BrdU 25 (1 day)	181	94	80	—	—
Tumor cells (P-1)	BrdU 25 (all the time)	237	207	242	—	—
Chemical induced	None	84	61	—	—	—
Tumor cells (P-3)	BrdU 25 (1 day)	101	88	—	—	—
	BrdU 25 (all the time)	143	193	—	—	—

[a] Counts per minute of incorporated [^{3}H]TMP per ml.
[b] Not done.

found in cultured guinea pig embryo, kidney, or spleen cells. However, subsequent to the BrdU or IdU treatment of the cultured cells, the similar particles were activated from all of the cultured normal guinea pig cells (Table I).

As previously described (7) numerous extracellular virus particles were seen in cultures treated with chemicals (Fig. 1). These particles

TABLE III. Reinducibility of Reverse Transcriptase Activity in Cultured Guinea Pig Cells by BrdU Treatment.[a]

Cells[b]	Passage number	Reverse transcriptase activity (cpm)	
		No BrdU	BrdU (25 μg/ml)
NP clone No. 12	1	89[c]	388
	2	76	453
	3	96	293
	4	86	284
	5	72	366
	6	88	366
	7	75	560
Kidney	1	72	350
	2	62	406
	3	58	297
Spleen	1	58	182
	2	63	283
	3	66	451

[a] After treatment of BrdU for 24 hr, cells were washed and fresh medium was added. At day 6 after treatment, fluids were examined for the presence of reverse transcriptase activity as described.
[b] Cells from NIH strain.
[c] Counts per minute of incorporated [^{3}H]TMP.

TABLE IV. Induction of Reverse Transcriptase from Guinea Pig Nonproducer Clone No. 12 Cells by Various Chemicals[a]

Chemical	Concentration (μg/ml)	Reverse transcriptase activity(CPM) at the day after exposure		
		7	9	14
None	—	61[b]	56	56
5-Iododeoxyuridine	100.0	482	160	75
5-Bromodeoxyuridine	100.0	477	162	68
5-Flurouracil	0.3	74	149	66
2-Amino-6-mercaptopurine	6.0	78	135	53
Cytochalasin B	12.0	76	65	53
3-Methylcholanthrene	1.0	60	117	49
7, 12-Dimethylbenz(*a*)anthracene	0.1	74	66	61

[a] After treatment of chemicals for 24 hr, cells were washed and fresh medium was added. At various intervals the medium was collected and virus particles were pelleted by centrifugation at 30,000 rpm for 2 hr. The pellets were resuspended in 1/100th of the original volume and assayed for viral enzyme.

[b] Counts per minute of incorporated [^{3}H]TMP per ml.

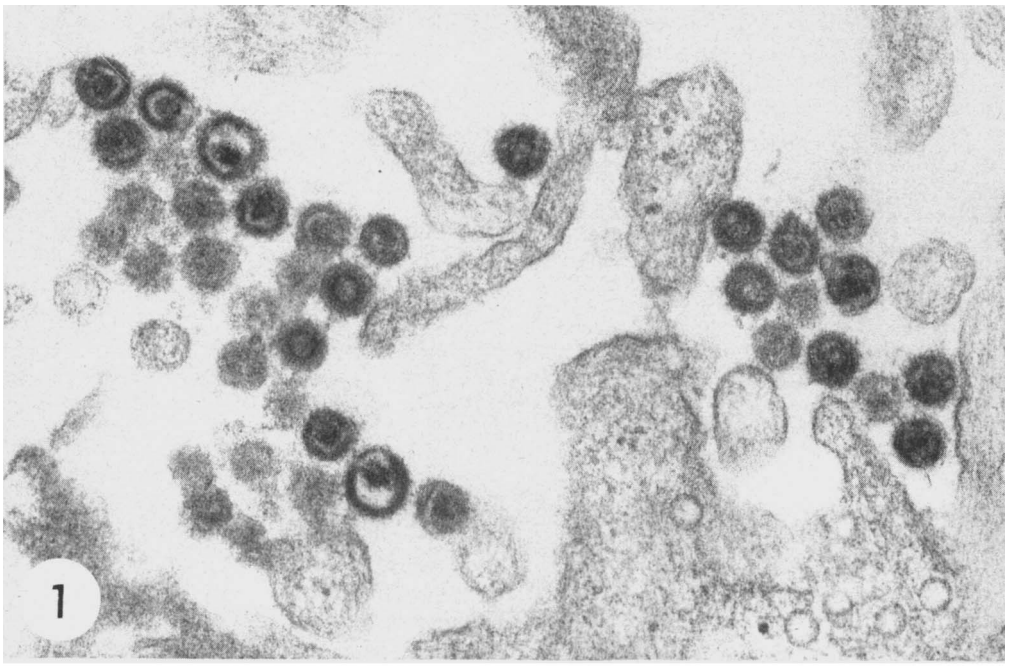
were approximately 100 nm in diameter, had a relatively thick poorly defined outer coat, and a dense nucleoid which was often slightly accentric. Immature extracellular forms were seen on occasion (Fig. 1). The entire immature particle was fairly dense, and the morphology was not clearly delineated. The inner component of the nucleoid was relatively translucent and measured approximately 50 nm in diameter. The outer coat was dense and fuzzy in outline but often displayed spike-like projections. Intracytoplasmic type A particles were frequently seen (Fig. 2) and on occasion could be seen budding off the cell surface presumably to form the free extracellular form. Intracisternal type A particles were rarely seen.

Biophysical and antigenic properties of induced virus. The NP clone 12 and kidney cells were grown for 24 hr in the presence of medium containing BrdU (25 μg/ml). The cultures were incubated for 8 days at 37° under 5% CO_2 in air. Fluids were tested for the release of particles by [^{3}H]-uridine labeling as described above. Sucrose gradient banding of supernatant from the BrdU-treated culture showed a peak of radioactivity at a buoyant density of approximately 1.16–1.17 g/ml. On the other hand, gradients of untreated cells showed no peak.

Antigenic characterization of induced virus was done in collaboration with Dr. R. V. Gilden. The banded virus was disrupted by Tween-80 ether and tested for reactivity in CF and gel diffusion tests with sera reactive with murine, hamster, rat, and feline leukemia virus, RD-114, woolly monkey virus gs antigens, and the gs-3 determinant using reagents and methods previously described (17, 18). No positive reactions were found. However, in absence of substantive data on the purity of the bands and polypeptide pattern of the disrupted virus no definitive conclusions are possible. The relationship of the viral polymerase to known type C polymerases was tested by antibody inhibition using sera to murine leukemia virus and RD-114 virus enzymes. Utilizing methods described previously (19, 20), no inhibition of the guinea pig viral enzyme was obtained. These tests were made using the synthetic template poly (rA:dT_{12-18}) and [^{3}H]-TTP as substitute. Activity was 10^3 cpm (background 200 rpm) with or without added antibody. The above data are consistent with the suggestion that this virus is not related to known Type C viruses, but much more work is needed before the relationship to the Type C family is clearly defined.

Growth of induced virus. An extensive effort was made to grow these induced viruses in various mammalian cells. So far, all the attempts have failed. Occasionally, cells infected with virus-containing supernatant fluids had a little higher level of polymerase activity than noninfected ones, but none showed cytopathogenic effect nor any virus particles were observed in the infected cells. Numerous direct and indirect rescue experiments by cocultivation were also nonproductive.

Discussion. The experimental results above showed that guinea pig leukemia-like virus can be activated not only from guinea pig NP lines but from cultured normal guinea pig cells.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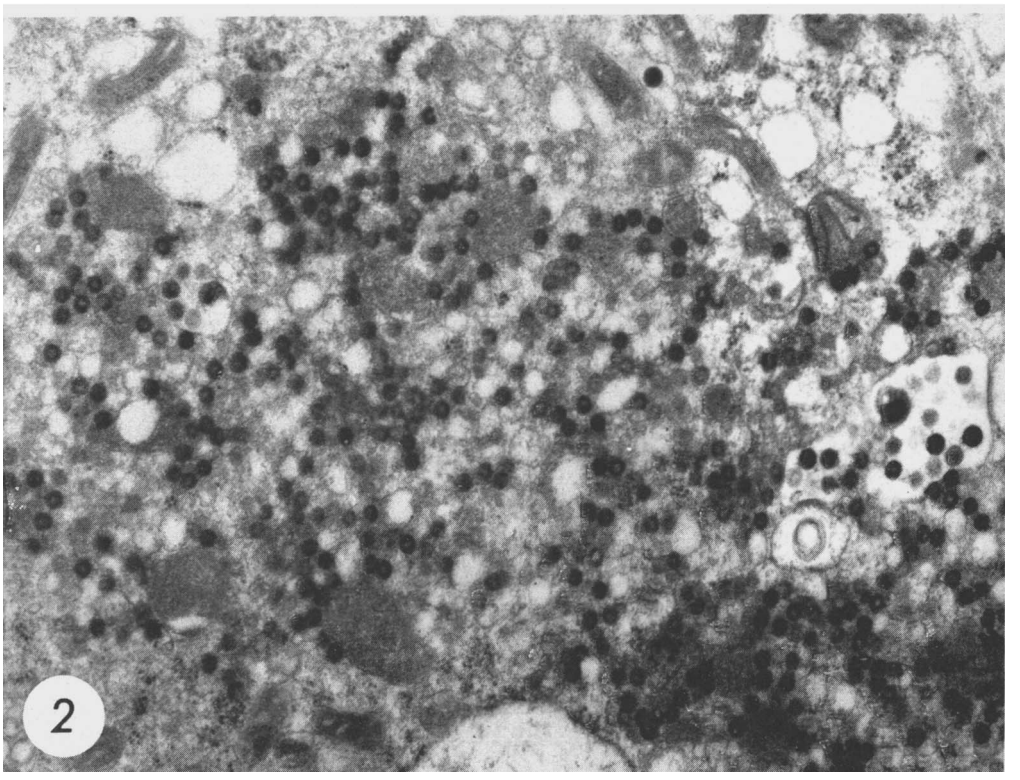
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RNA-dependent DNA polymerase activity was induced after exposure of cells to chemicals. The extracellular mature particles were similar to those observed in guinea pig leukemia (1–4) and had a density of 1.16–1.17 g/ml. Most of intracellular particles were located in the cytoplasm rather than the intracisternal location previously reported for guinea pig leukemia (1, 2, 4). Feldman and Gross (4) also described particles within the cisternal of the endoplasmic reticulum resembling intracisternal "A" particles. Indeed, the particles we observed in these cells from cultured guinea pig cells bear more morphological similarity to type B particles than to type C particles. In banded virus prepared from cells after BrdU treatment (at the time of virus production), no type C virus antigen against mouse, rat, feline, hamster leukemia viruses, RD-114 virus and woolly monkey virus was detected by CF and gel diffusion tests. No inhibition of the viral enzyme was obtained when the guinea pig viral polymerase was tested by antibody inhibition using sera to murine and RD-114 enzymes.

Activation of viruses from cultured guinea pig cells by chemical treatment has recently been reported. However, the morphological identity of the particles found in the cultured cells after chemical treatment is controversial. Hsiung (5) has shown that activation of virus particles was accomplished by exposure of primary and passaged cells, derived from leukemic and normal guinea pigs. Numerous particles considered to be type C were seen in intracytoplasmic vacuoles and in extracellular spaces. The particles had a density of 1.17 g/ml in sucrose gradient but devoid of reverse transcriptase activity (21). Nayak and Murray (6) also found the particles morphologically resembling type C virus after BrdU treatment of cultured guinea pig cells. Their particles had the density of 1.16 g/ml and possessed oncornavirus-specific reverse transcriptase activity.

BrdU treatment induced virus particles in all the culture of normal or virus-free clonal and leukemic guinea pig cells tested to date. Cells of strain 13 guinea pigs were also found susceptible to BrdU induction. Thus, guinea pig leukemia-like virus, potentially inducible from any guinea pig cells, appears to be truly endogenous virus. Nayak *et al.* (22) studied recently its expression in BrdU-treated and in leukemic guinea pig cells and found that the same amount of viral DNA was present in leukemic cells as in normal or BrdU-induced cells, suggesting that the particles seen in leukemic cells resulted from the expression of endogenous viral DNA. It had already been shown that type C viruses can be activated in virus-free cells of three species, the mouse, rat, and cat *in vitro* by the presence of BrdU or IdU and that the chemically induced viruses were endogenous helper type C virus of the host cells rather than any helper virus of the exogenous virus (16, 23–26). More recent reports indicate that the characteristic type C virus can be activated in the tissue cultures isolated from a pulmonary adenocarcinoma with IdU (27, 28). Together with our findings and those reported with murine leukemia sarcoma viruses (16, 23–26), it would seem that chemical induction may be extremely useful for the activation and enhancement of certain RNA tumor viruses in normal and malignant cells of man and animals. The mechanism by which chemical compounds induce viruses from normal or virus-negative clonal cells remained to be clarified. Lowy *et al.* (29) and Teich *et al.* (15) have reported that incorporation of BrdU or IdU into cellular DNA plays a vital role in the activation of murine leukemia virus production in the AKR cell lines. Experimental evidence supports also that BrdU or IdU may at least in some cases, activate an unexpressed or partially expressed cellular gene cording for virus-specific information (30).

Attempts to infect uninduced cells *in vitro* or induced leukemia *in vivo* by chemically induced guinea pig viruses or by the cells releasing virus particles after chemical treatment have not yet proven successful.

Summary. Particles resembling guinea pig leukemia virus were activated from guinea pig nonproducer cells and cultured normal guinea pig cells after chemical treatment. These particles were approximately 100 nm in the mature form, had a density of 1.16–1.17 g/ml, and contained RNA-dependent DNA polymerase activ-

FIG. 1. Extracellular mature and immature particles resembling guinea pig leukemia virus seen in BrdU-treated guinea pig spleen (NIH strain) cells. (× 82,500).

FIG. 2. Particles resembling guinea pig leukemia virus observed in BrdU-treated guinea pig spleen (NIH strain) cells. Intracytoplasmic type A particles were also seen (× 32,500).

ity. The peak of polymerase production was observed between 6 and 8 days after 1-day bromodeoxyruidine (BrdU) treatment. Polymerase activity was reinducible and was always detectable in the BrdU-containing medium of the cultures in prolonged cultivation. The results of screening various chemicals demonstrated that thymidine analogs (BrdU and 5-iododeoxyuridine) were by far the most efficient inducers of guinea pig leukemia-like virus in the cells. Antigenic relationship to known type C viruses has been discussed.

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