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Characteristics of and Relationship Between C Particles and Intracisternal A Particles in Cloned Cell Strains

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Four murine tissue culture cell strains, which originated by cloning from one common cell of subcutaneous connective tissue origin, were examined for the presence of virus by electron microscopy and complement fixation techniques. The relative distribution of C particles and intracisternal A particles was determined. Thereafter, the characteristics of and relationship between A- and C-type particles were investigated. Cell extracts were passaged onto virus-free Swiss and C3Hf mouse embryo tissue cultures; CF and EM tests were again made to investigate the infective capacities of the various particle types. Although C particles were found in passage cultures exposed to extracts from the C particle-bearing strains, no A particles were found in any passage culture. These results indicate that the intracisternal A particle was neither infective nor developmentally associated with the C particle. A positive CF test was correlated with the presence of morphologically detectable C particles and was independent of the concentration of A particles.

The intracisternal A particle has been an enigma up to the present time. Mistakenly introduced to the literature in 1955 as the elementary body of the anopheles A virus (18–20), renamed in 1958 (3, 5), subclassified and subdistinguished from the intracytoplasmic form of the mammary tumor virus in 1962 (8), it was again reclassified in 1966 (2). In this paper we are dealing with this latter particle: an electron-dense doughnut-shaped intracytoplasmic particle which has two shells (70 and 30 mμ in diameter) and develops within the cisternae of the endoplasmic reticulum. Although consistently found in Ehrlich ascites tumor cells (1, 18, 40), plasma cell tumors (11, 28, 33, 34), clone 929-L cells (4, 6, 29), methylcholanthrene-induced sarcomas (12, 21), melanoma S91 (9), and various leukemia neoplasms (10, 13, 14, 27), the A particle has never conclusively been demonstrated to possess any biological activity, and thus recurs in the literature as a virus-like particle which "may be a by-product of viral activity or the result of nonspecific cellular damage" (16). It has likewise been suggested that the particle might be a precursor or immature form of the C particle with which it is frequently associated and which is known to be viral. Recent attempts at isolation and biochemical analysis of the A particle have met with some success (30) but two important questions still remain unanswered. (i) Is this "virus-like" A particle the precursor of, or at all related to, the C particle? (ii) Is the A particle infective?

In a previous study employing electron microscopy and complement fixation (CF) tests (24), several mouse cell lines and strains were investigated for the presence of leukemia-type viruses. Eight of the strains, derived in vitro from the same cell and subsequently recloned, possessed both A- and C-type particles in various quantities. The combination of the two testing methods and the cloned cell lines seemed to offer an opportunity for a biological approach to the riddle of the A particle.

MATERIALS AND METHODS

History of the cell strains studied. Several years ago, an effort was made to study the incidence of "spontaneous" neoplastic transformation in 17 cell populations derived in vitro from one cell isolated from normal subcutaneous connective tissue of an adult C3H/He mouse (36–39).

More recently, eight cell strains derived from the original clone (Fig. 1) were examined for CF leukemia antigens and recoverable virus, and were examined by electron microscopy for both C and A particles (24). From these strains, four were selected for the present study.

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Detection of murine leukemia virus (MuLV) and MuLV group reactive antigen. The general procedures for CF antigen and tissue culture virus recovery testing were as previously described (26). Briefly, the cells from five T-15 flasks were pooled and resuspended in 5 ml of supernatant fluid. A small portion was centrifuged, the cells were resuspended in one-fifth volume of fluid and sonically treated. These samples were titrated in the CF test against four units of broadly reactive rat antiserum for the MuLV group. The remainder of the suspension was quick-frozen and thawed and inoculated in 0.2-ml amounts onto four plate cultures of 1-day-old NIH Swiss mouse embryo tissue cultures (NIH-METC). After maintenance for 19 to 21 days with twice-weekly fluid changes of Eagle's minimal essential medium with fetal bovine serum (10%), antibiotics, and glutamine, control and inoculated cultures were harvested for electron microscope observation, CF antigen testing, and passage to fresh NIH-METC.

Electron microscopy. Cells were scraped from culture flasks and centrifuged in an IEC rotor no. 269 for 10 min at 1,500 rev/min. Pellets thus formed were cut into small pieces, fixed in 1% chrome-osmium (7) for 1 hr, rinsed twice in aqueous uranyl acetate (*p*H 5.0), then transferred to fresh uranyl acetate for 90 min. After rapid alcohol and propylene oxide dehydration, the tissue was left overnight in propylene oxide and Epon-Araldite (1:1), and finally embedded in Epon-Araldite (31). Sections were cut on a Reichert OM U2 ultramicrotome with a diamond knife, stained with uranyl acetate and lead citrate and viewed in a Siemens Elmiskop IA electron microscope. Two grids from five blocks of each pellet were examined for the presence of particles. At each passage level, controls of uninoculated NIH-METC were also sectioned and observed.

RESULTS

When the eight cell strains were first studied as part of a combined CF-electron microscope investigation of several murine cell lines (24), all were found to possess intracisternal A particles and usually budding or mature C particles, or both (Fig. 2–5). To test the relationship between these two particle types, four cell strains (NCTC 3069, 4953, 2472, and 4700), representative of tumor-derived cultures, high-tumor producing and low-tumor producing strains (Fig. 1) were again examined several months and several culture generations later by electron microscopy and by CF. The only significant difference from the earlier test was that NCTC 3069 was now conclusively CF-positive and contained a large number of mature and immature C particles.

In general, the A particles were found in expanded cisternae of the endoplasmic reticulum (ER) or budding into the cisternae from a ribosome-free portion of the ER membrane (Fig. 3, 4), but they were also seen in the perinuclear space. Usually round to slightly oval, the particles

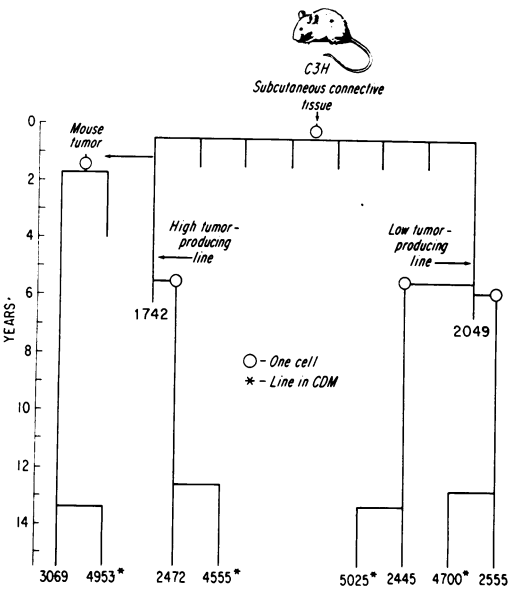


FIG. 1. *Outline of development on various culture fluids of four high tumorigenic cell strains and four low tumorigenic cell strains originating from one cell of normal subcutaneous connective tissue of an adult C3H/He male mouse. CDM = chemically defined medium, NCTC 135, without any serum protein or hydrolysate supplement (17). Cells of the other strains examined in this study were grown in NCTC 135 supplemented with 10% horse serum.*

averaged about 75 to 80 mμ in diameter with a 30 to 35 mμ electron-lucent center. In micrographs with good resolution, the unit membrane could still be observed around the edge of the particle, with an electron-dense region approximately 20 to 25 mμ wide closely apposed to it (Fig. 3, 4). This dense region was delimited by the unit membrane and a more heavily staining area which surrounded the electron-lucent center.

The C-type particle clearly distinguishable from the A particle in size, structure, and location was found both budding from the plasma membrane and in the medium of C particle-producing cultures (Fig. 5). Approximately 100 mμ in diameter, the C particle resembled in both its mature and immature form (Fig. 5) the particle often associated with murine leukemia and described by several investigators. Yumoto et al. (41) recently reviewed the literature on this subject.

Table 1 shows results of the original CF and electron microscope tests on the four cell strains. The type and average number of particles found per thin section of cells in 25 distinct electron microscope fields are listed in the last three columns. The presence of MuLV group-specific

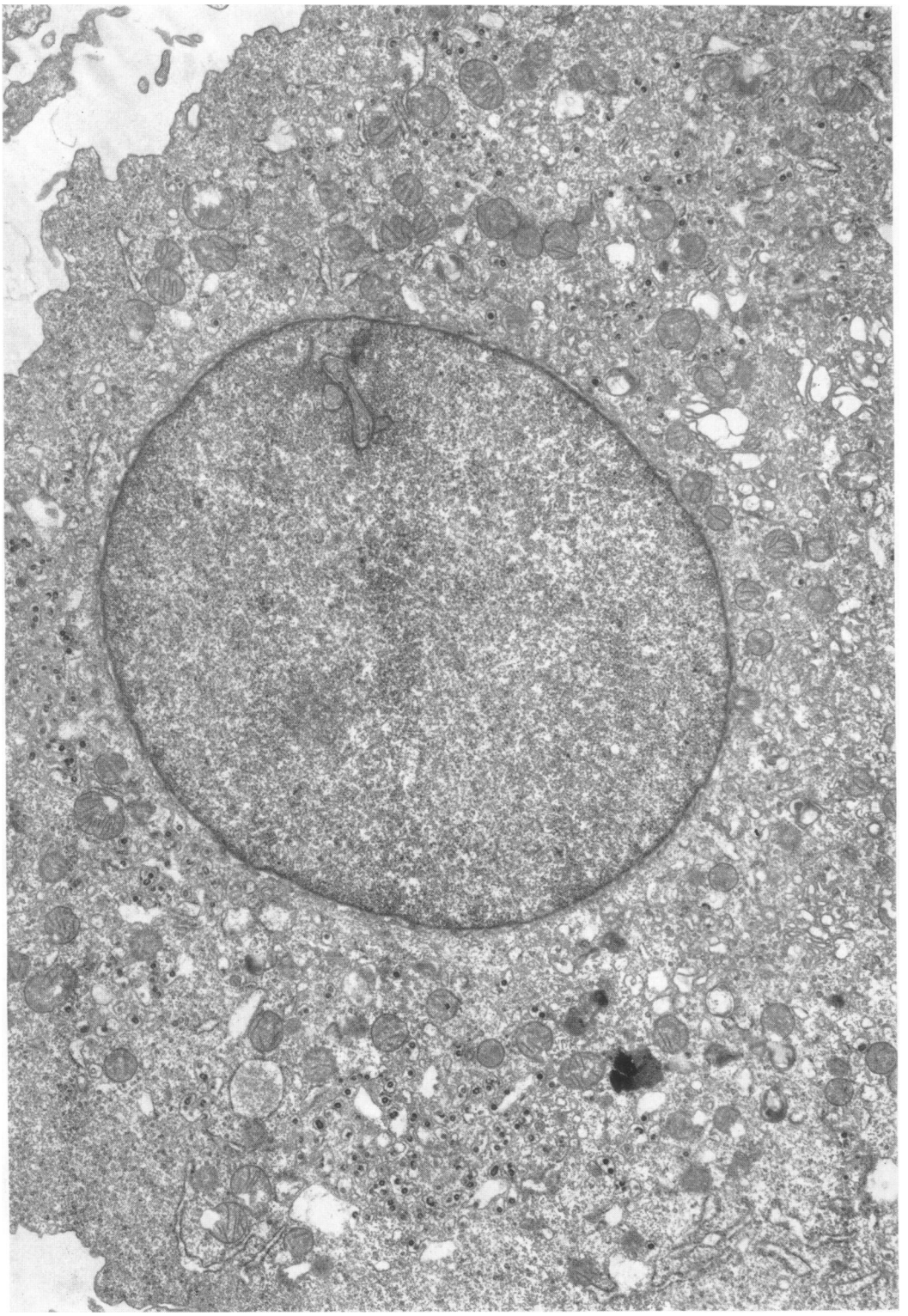
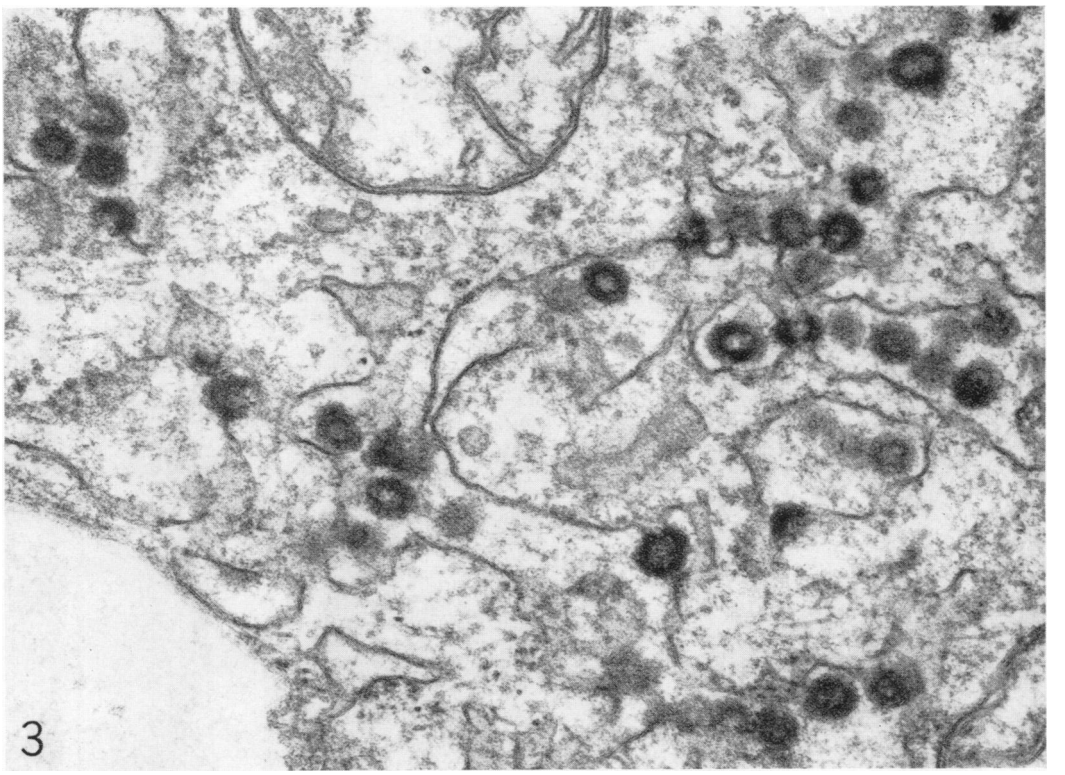


FIG. 2. *Micrograph of a cell from NCTC 4953, showing the distribution of intracisternal A particles throughout the cytoplasm.* × *28,000.*



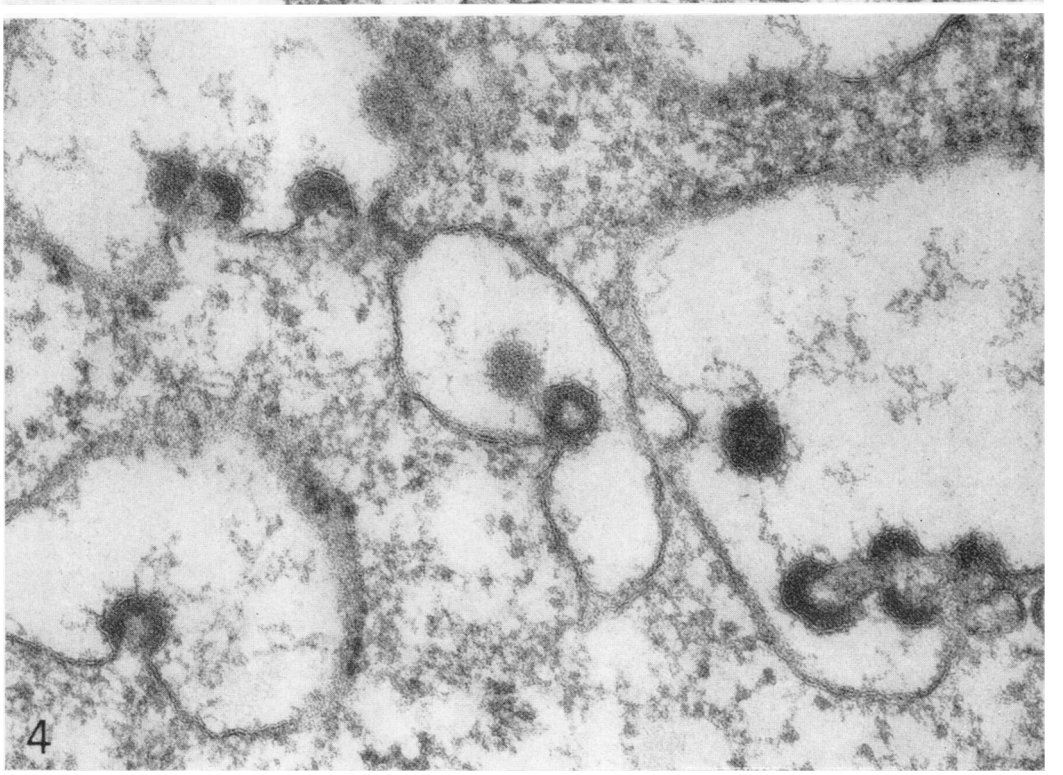


FIG. 3. *A particles in intracisternal spaces in cell of NCTC 3069. Space is formed by expansion of rough endoplasmic reticulum.* × *70,000.*

FIG. 4. *A particles budding from segment of rough endoplasmic reticulum in cell of NCTC 4700. Unit membrane is still visible around outer edge of particles.* × *105,000.*

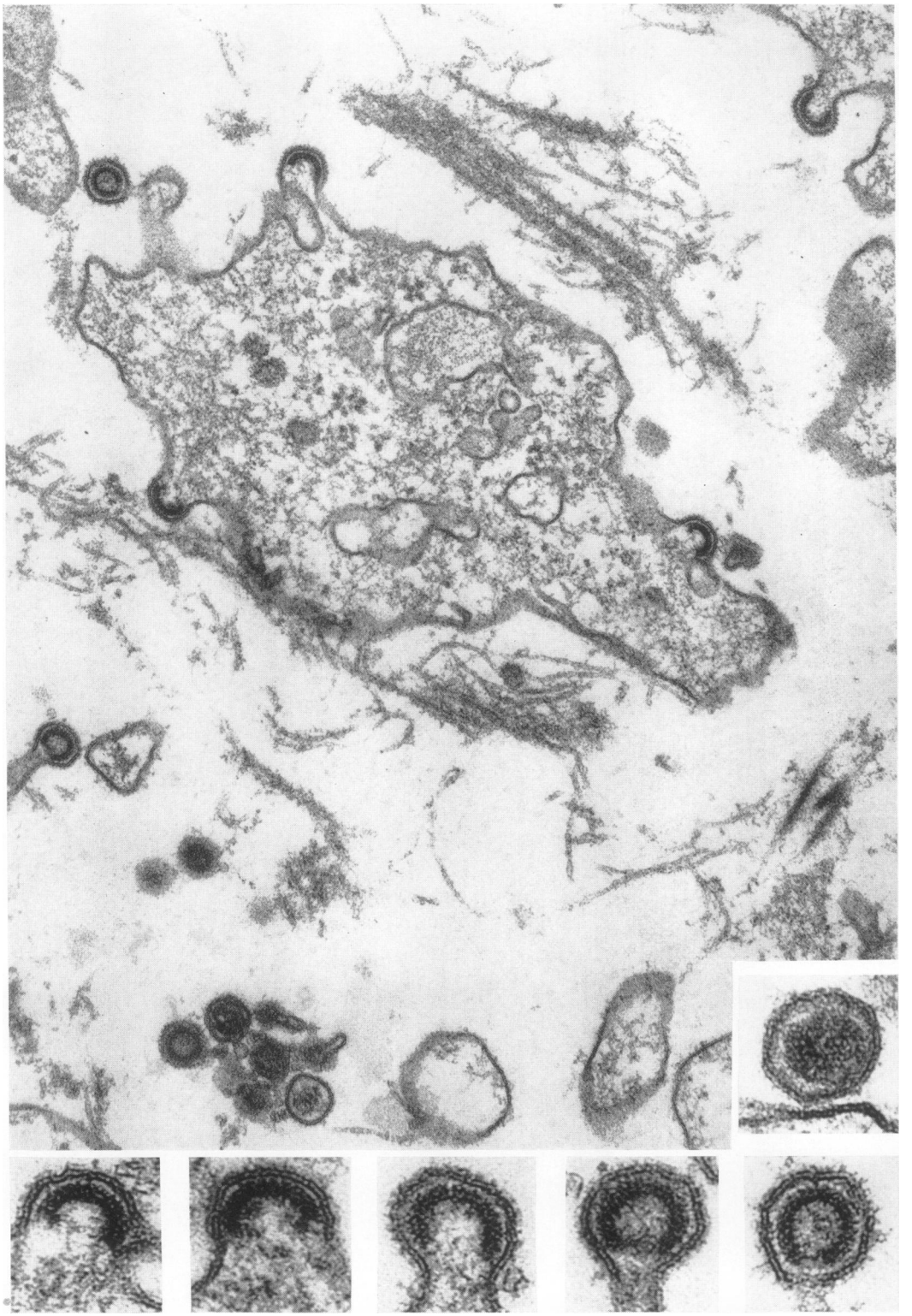


FIG. 5. *Cells from Swiss mouse embryo tissue culture (NIH-METC) after exposure to extract from NCTC 3069. Budding and mature C particles are visible. × 70,000. Inset: × 180,000.*

antigen was correlated with the presence of mature C particles, and was independent of the concentration of A particles.

To check the infectivity of particles occurring in the cell lines, three successive passages on NIH-METC of frozen and thawed suspension from the four respective strains were tested by electron microscopy and CF. As Table 2 indicates, all passage cultures originally inoculated with extracts of 3069 and 2472 cells were CF-positive and exhibited morphologically identifiable C particles, both mature and immature (Fig. 5). Similar particles had been detectable in the direct test of these cell strains. However, in cultures which were CF-negative (NCTC 4953 and 4700) in the direct test, and in which budding and mature particles had been minimal or absent, no C particles could be found in the subsequent passages, which consistently remained CF-negative. Here, again, the concentration of A particles in the starting cultures had no effect on the presence of C particles or the outcome of the CF test. Thus, although NCTC 4953 and 4700 have continued to possess large numbers of A particles (Fig. 2), NIH-METC treated with frozen and thawed suspensions of these cultures remained CF-negative. A particles were found neither in passage cultures exposed to suspensions from high A particle lines nor in those exposed to suspensions from low A particle lines. This was true whether the passage cells possessed or lacked C particles. In all controls thus far examined, untreated NIH-METC were free of A and C particles and were repeatedly CF-negative.

Pellets from the medium of a CF-negative second-passage culture, inoculated originally with an extract from 4953 cells, were compared with those from a CF-positive second-passage culture derived from 2472. In the former, no C particles were visible (Fig. 6), whereas several were present in the latter (Fig. 7).

Cells from NCTC 4953 and NCTC 2472, respectively, were implanted subcutaneously into weanling C3Hf mice, and the resulting fibrosarcomas were examined with the electron microscope. Portions of the tumors were put back into tissue culture and likewise surveyed with the electron microscope. The particle distribution of

TABLE 1. *Comparison of concentration of A- and C-type particles (per thin section of 25 distinct cell sections), murine leukemia virus (MuLV) antigen, and recovery of viruses of MuLV family from four mouse embryo tissue culture cell strains*

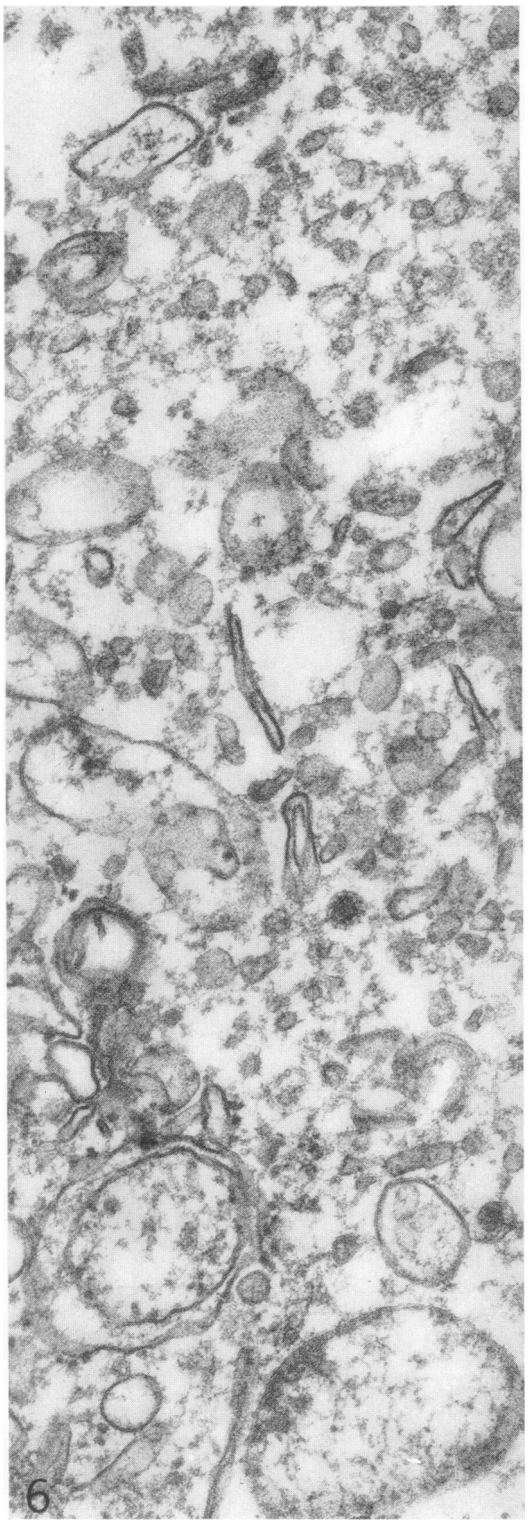
Cell strain	Medium	Complement-fixing (CF) antigen		Particles/cell section, direct test (avg no.)		
		Direct	Passage	A	C-budding	C-mature
3069	NCTC 135 + 10% H.S.	−	+[a]	14.5	0.4	0.3
4953	NCTC 135	−	+	18.2	1.0	0.0
2472	NCTC 135 + 10% H.S.	+	+	2.5	0.7	11.0
4700	NCTC 135	−	−	16.3	0.2	0.5

[a] Plus (+) indicates development of MuLV group-reactive CF antigen in NIH-METC inoculated with cell suspensions of test lines, indicating replication of C-particle virus of the MuLV family.

TABLE 2. *Comparison of relative concentrations of A- and C-type particles, murine leukemia virus (MuLV) complement-fixing (CF) antigen, and recovery of viruses of the MuLV family from four cloned mouse embryo tissue culture cell lines*

Cell strain	CF-antigen[a] (direct test)	Relative[b] concn of particles (EM)	Passage in Swiss mouse embryo cells						Passages on C3H embryo cells	
			First		Second		Third			
			CF-antigen	EM particle concn	CF-antigen	EM particle concn	CF-antigen	EM particle concn	CF-antigen	EM particle concn
3069	>16	C = C_B > A	16	C, C_B	16	C, C_B	8	C, C_B	2	C, c_B
4953	<2	A > c, c_B	<2	—	<2	—	<2	—	<2	—
2472	>16	C > C_B > a	8	C_B > C	16	C, C_B	NT		4	C, C_B
4700	(2)	A > c_B	<2	—	<2	—	<2	—	<2	—

[a] Reciprocal of CF-antigen titer in sonically treated cell packs; parenthesis indicates partial reactivity.
[b] A = intracisternal A particle, C = C particle, C_B = budding C particle. Lower case letter = very small number of particles; NT = no test done. EM = electron microscope.



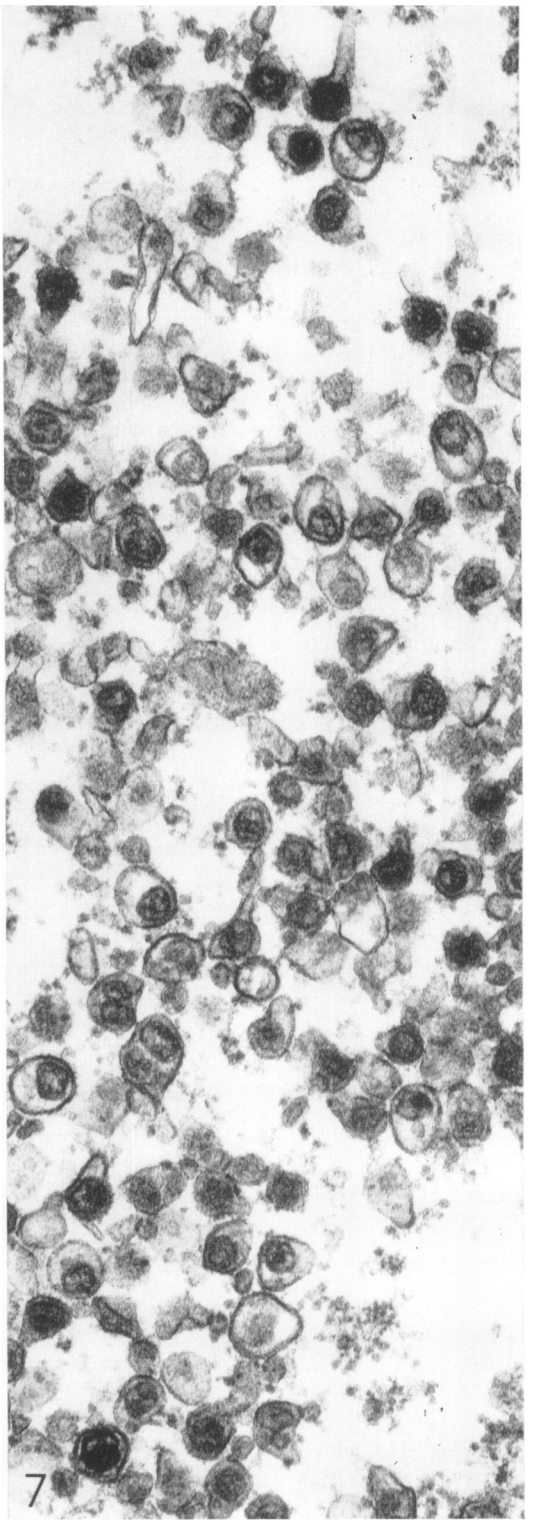


FIG. 6. *Representative section through pellet prepared from medium of C3Hf mouse embryo tissue culture (NCTC 5143) previously exposed to extract from NCTC 4953.* × *70,000.*

FIG. 7. *Section through pellet prepared from medium of C3Hf mouse embryo tissue culture (NCTC 5143) previously exposed to extract from NCTC 2472.* × *70,000.*

the initiating cells was retained through the tumor and back into tissue culture. NCTC 4953 was characterized both in vitro and in vivo by a marked predominance of A particles, whereas in NCTC 2472, A particles were scarce and C particles bountiful.

On the chance that a particle from C3H mouse might not grow on Swiss mouse tissue cultures, or that growth might be more efficient on syngeneic cells, passage of suspensions from the four cell strains was made to cells from an established C3Hf-derived culture line, NCTC 5143. This line had been CF-negative and free of electron microscope-detectable virus particles in previous tests. Table 2 shows results identical with those of Swiss mouse embryo tissue culture passages: no A particles visible in any of the cultures, and C particles present in NCTC 3069 and 2472. A previous study (22, 23) had shown that a similar C-type particle isolated from NCTC 4705, also derived from C3H mouse cells, actually grew better in NIH-METC than in the C3H cells of origin.

Because of the morphological similarity of these C particles to known leukemia agents and their positive response to broadly reactive antileukemia virus rat sera, tumor extracts of C particles prepared from NCTC 2472-initiated tumor were given to Robert Ting (*personal communication*), who demonstrated their ability to function as helper (25) in the recovery of murine sarcoma virus (Moloney). Injection of similar extracts into newborn mice failed to produce any signs of leukemia after more than 15 months in vivo.

DISCUSSION

As far as can be determined by electron microscope examination, A particles in extracts prepared from particle-rich cells are unable to initiate a replicative cycle in NIH Swiss- or syngeneic-strain tissue culture cells. systems which support the growth of viruses of the murine leukemia group. On the basis of the frequent association of A- and C-type particles in a variety of tumors and cell lines, a dependence on C particle replication for growth of an A particle-related virus could be postulated. However, even in the presence of infective murine leukemia group virus and corresponding C particles, as in lines 3069 and 2472, no evidence for A-particle replication was found by the testing procedures used. Several other independent investigations (11, 33–35) have likewise failed to demonstrate biologically active A particles.

If previous speculation (3, 33, 34), that the A particle is, indeed, a variant form or developmental stage of the C-type, had been true, then one would statistically expect to find (especially in passage cultures heavily infected with budding and mature C particles) an occasional A particle. This, however, was not the case. Regardless of C particle titer, A particles were not to be found.

Dales and Howatson (6) and, more recently, Kindig and Kirsten (29) have reported morphologically similar, but nontransmissible, C particles in established cell lines. That the C particle in the present study is biologically active, there is no doubt, although injection of C particle preparations from NCTC 2472 and 2049 into susceptible newborn mice has failed to induce leukemia after more than 1 year in vivo.

Several cell strains possessing a large number of A particles have remained negative in the direct test for MuLV CF antigen. Failure to detect antigen indicates only that the antisera used, selected for reactivity with MuLV family, C particle associated envelope antigens, do not contain detectable levels of antibody directed against A particles or any antigenic byproducts. In an ultrastructural and immunological study on primary methylcholanthrene-induced murine sarcomas, de Harven (12) found that in tests for G (Gross) antigen, by adsorption of cytotoxic G antibody (32), the A particles "either escape serological detection due to their internal localization or belong to an unrelated group of agents."

Almost as elusive as the characterization of the A particle has been its classification, and the use of identical terms for different entities has added to the chaos. de Thé (15) aptly summarized the state of the A particle: "When one realizes that type A1 described by de Harven means immature C particles for Dalton, one can easily realize a source of confusion. Moreover, Dalton described as A1 particles the intracytoplasmic particles, budding from the endoplasmic reticulum in the plasma cell tumors in mice, whereas A1 particles of de Harven represent extracellular virions." As a result of the recently proposed classification of oncogeneic ribonucleic acid viruses (2), much of the confusion of terms has disappeared. However, in spite of this clarification and the present investigation showing A particles to be noninfectious under conditions which readily demonstrated infectivity for C particles, two fundamental problems still remain: if the particle is not viral, what causes it to proliferate, and what is it?

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